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Methanogenic crude oil-degrading microbial consortia are not universally abundant in anoxic environments

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ABSTRACT

Crude oil-amended microcosms were prepared with inocula from eleven anoxic environments (4 river sediments, 3 lake sediments, and 4 sludges from wastewater treatment reactors) to determine their ability to produce methane from the biodegradation of crude oil. Over incubation periods of up to 1150 days, oil-stimulated methanogenesis and concomitant loss of alkanes occurred in microcosms prepared with five of the inocula whereas six of the inocula did not show oil-stimulated methane production. Bacterial and archaeal communities from microcosms exhibiting high levels of oil-stimulated methanogenesis were distinct from communities where methanogenic crude oil degradation was not detected. Successional changes were consistent with the quantitative enrichment of syntrophic hydrocarbon degrading bacteria and methanogens over time. In conclusion, in oil-impacted environments methanogenic crude oil-degrading microbial consortia are present in relatively low abundance and exhibit slow growth, and while they may be ubiquitously distributed they may not be present at sufficiently high abundance to be detected.

1. Introduction

It is well documented that *n*-alkanes, a major fraction of most crude oils, are biodegradable under methanogenic conditions when provided as single, pure compounds (Anderson and Lovely, 2000; Zengler et al., 1999) and in crude oil and other complex mixtures (Berdugo-Clavijo and Gieg., 2014; Clothier and Gieg, 2016; Gieg et al., 2008, 2010; Gray et al., 2010, 2011; Jones et al., 2008; Li et al., 2012; Liang et al., 2015; Mbadinga et al., 2011; Sherry et al., 2010, 2014; Siddique et al., 2006, 2011; Townsend et al., 2003; Zhou et al., 2012). Slow methanogenic biodegradation of hydrocarbons over geological timescales is responsible for the formation of heavy oils in subsurface petroleum reservoirs (Jones et al., 2008; Gieg et al., 2008; Bennett et al., 2013), and methanogenesis has been shown to be an important process of natural attenuation in hydrocarbon contaminated aquifers (Dojka et al., 1998; Feisthauer et al., 2012) and soils (Kasai et al., 2005; Sarkar et al., 2005).

Methanogenic biodegradation of hydrocarbons has been shown to proceed through the concerted action of fermentative bacteria together with methanogenic archaea (Gieg et al., 2014; Gray et al., 2011; Jones et al., 2008; Mbadinga et al., 2011; Zengler et al., 1999). *Deltaproteobacteria* from the genus *Smithella* are significant in the degradation of crude oil *n*-alkanes in syntrophic partnerships with hydrogenotrophic (Gray et al., 2011; Sherry et al., 2014; Toth and Gieg, 2018) and acetoclastic (Ding et al., 2015; Embree et al., 2014; Zengler et al., 1999) methanogens. Methanogenic alkane degradation using CO_2 as an electron acceptor to produce H_2, which is then utilised by methanogens to produce CH_4, has been implicated as the dominant methanogenic degradation pathway for in-reservoir crude oil biodegradation (Jones et al., 2008; Gray et al., 2011; Jimenez et al., 2012). A number of studies have also identified *Smithella* and *Syntrophus* in the family *Syntrophaceae* as dominant microorganisms in hydrocarbon impacted anoxic soils, sediments, aquifers and deep groundwater associated with coal seams (Allen et al., 2007; Bakermans and Madsen, 2002; Dojka et al., 1998; Gründger et al., 2015; Kasai et al., 2005; Shimizu et al., 2007; Zengler

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et al., 1999).

The onset of methanogenic alkane degradation is often preceded by an extended lag phase which has been shown to last hundreds of days for methanogenic degradation of hydrocarbons in mature oil sands tailings (Siddique et al., 2011), in hydrocarbon-amended estuarine sediments (Jones et al., 2008; Sherry et al., 2014) and in anaerobic digesters treating hydrocarbon-contaminated wastewater and soil (Scherr et al., 2012). Significant amounts of methane production in enrichment cultures supplemented with long chain *n*-alkanes (nC_{15}-nC_{20}) was not observed until approx. 250 days after incubation at 55 °C (Zhou et al., 2012) and not until after 157 days in a study where the temperature was not specified (Liang et al., 2015). The slow onset of methanogenesis has been associated with a decrease in the hydrogen content in the enrichment cultures from ~2.2 μmol to ~0.6 μmol H_2 (Zhou et al., 2012), consistent with complete conversion of *n*-alkanes to methane only being thermodynamically feasible if methanogenic intermediates are maintained at very low concentrations (Dolfing et al., 2008, 2009). By contrast, a lag phase of only tens of days was observed in highly acclimated methanogenic enrichments degrading residual oil in sandstone core material (Gieg et al., 2008). The long lag periods observed result from slow growth (doubling times >30 days) of populations of the primary hydrocarbon degrading bacteria which are present in inocula at very low abundance (Gray et al., 2011).

Given the relatively low abundance and slow growth rates of methanogenic crude oil-degrading microbial consortia, we hypothesised that they may not be ubiquitously distributed or, at least they are not present in many environments in sufficiently high abundance to be detected even in prolonged laboratory incubations. To test this hypothesis, anoxic oil-amended microcosms were prepared with inocula from eleven anoxic environments (4 river sediments, 3 lake sediments, and 4 sludges from wastewater treatment reactors) and their ability to produce methane from the biodegradation of crude oil was determined. Analysis of bacterial and archaeal 16S rRNA genes was performed on oil-amended microcosms and on control microcosms to which no oil was added to establish whether there were consistent changes in the bacterial and archaeal communities during oil-stimulated methanogenesis.

1.2. Materials and methods

1.2.1. Inocula for methanogenic microcosms

Eleven different sources of inoculum were sampled for the preparation of methanogenic microcosms in order to assess the ubiquity of methanogenic hydrocarbon-degrading consortia in a diverse range of environments (Table 1). Microcosms were prepared with sediment from four rivers (River Tyne, River Wear, River Tees and River Coquet), and sediments from a hypereutrophic lake (Priest Pot), an oligotrophic lake (Buttermere) and a eutrophic lake (Rydal water). Sediments were collected from noticeably anaerobic zones at a depth of ~30 cm, filled to the brim of sterile vessels to minimise ingress of air, and stored at 4 °C for a maximum of 6 days prior to use in microcosms. Sludge from an anaerobic digester treating municipal waste (Newton Aycliffe) was used as inocula in methanogenic microcosms, as well as sludges from two aerobic digesters treating oil-refinery waste (Grangemouth and Humberside) which were incubated under anoxic conditions (Table 1). Sludge from an upflow anaerobic sludge blanket (UASB) reactor treating wastewater from the personal care industry obtained from a factory in Poland, which was previously used to investigate waste-to-energy opportunities (Ahammed et al., 2014), was also used as inocula (Poland, Table 1). Sludge inoculum were stored at 4 °C prior to use.

1.2.2. Preparation of methanogenic microcosms

Methanogenic microcosms (120 ml glass serum bottles (Wheaton, VWR)) containing 100 ml aqueous/sediment slurry with 20 ml headspace) were prepared with carbonate-buffered medium (Widdel and Bak, 1992) under anoxic conditions as described previously (Jones et al., 2008; Sherry et al., 2010). Salinity was adjusted to a brackish medium for river sediments (River Tyne, River Wear, River Tees and River Coquet, 7 g/L NaCl, salinity 17 ppt) and a freshwater medium for lake sediments and sludges (Priest Pot, Buttermere, Rydal water, Newton Aycliffe, Grangemouth, Humberside and sludge from Poland, 1 g/L NaCl, salinity 4 ppt). Salinity in microcosms at the start of the experiment (Day 0) was determined from the measured chloride ion content as a proxy, where salinity (ppt) = 0.00180665 Cl– (mg/L) (Table 1). Oil-degrading microcosms were amended with a North Sea light crude

Table 1
Site location, source and initial salinity of inoculum used in the preparation of methanogenic microcosms.

Location	GPS co-ordinates	Inoculum source	Sediment/sludge in microcosms (g)	Chloride (mg/L)	Salinity (mg/L)	Salinity (ppt)
River Tyne	54°57′51.15″N 1°40′59.30″W	River sediment	6.94 ± 0.08	9518 ± 611	17,195 ± 1103	17 ± 1.1
River Wear	54°53′52.28″N 1°28′44.04″W	River sediment	8.73 ± 0.66	8412 ± 1204	15,197 ± 2175	15 ± 2.2
River Tees	54°35′6.59″N 1°13′36.75″W	River sediment	6.69 ± 0.55	11,829 ± 1724	21,370 ± 3114	21 ± 3.1
River Coquet	55°20′53.13″N 1°36′29.21″W	River sediment	9.47 ± 0.31	10,698 ± 1396	19,327 ± 2522	19 ± 2.5
Newton Aycliffe	54°48′16.34″N 1°34′55.24″W	Anaerobic digester sludge treating municipal waste	7.96 ± 0.31	2205 ± 119	3984 ± 215	4 ± 0.2
Poland	Not available	Sludge from a UASB reactor treating personal care industry wastewaters	7.20 ± 0.28	2170 ± 22	3920 ± 41	4 ± 0.04
Grangemouth	56° 1′6.80″N 3°44′20.74″W	Aerobic sludge treating oil refinery waste	6.22 ± 0.61	2264 ± 233	4089 ± 422	4 ± 0.4
Humberside	53°38′2.99″N 0°11′54.12″W	Aerobic sludge treating oil refinery waste	8.57 ± 0.36	1982 ± 209	3581 ± 377	4 ± 0.4
Priest Pot	54°22′13.51″N 2°59′42.25″W	Lake sediment (hypereutrophic)	6.86 ± 0.32	2578 ± 31	4658 ± 56	5 ± 0.1
Buttermere	54°32′30.04″N 3°16′29.54″W	Lake sediment (oligotrophic)	7.92 ± 0.26	2556 ± 21	4617 ± 38	5 ± 0.04
Rydal Water	54°26′47.63″N 2°59′30.60″W	Lake sediment (eutrophic)	7.72 ± 0.38	2161 ± 256	3904 ± 463	4 ± 0.5

oil (250 mg). The oil was undegraded (Peters and Moldowan PM 0; Peters and Moldowan, 1993, Type II kerogen sourced oil), with an API gravity of ~38°. Oil-free control microcosms were prepared to determine the extent of methanogenesis in the absence of the addition of crude oil. Inhibited control microcosms containing bromoethanesulphonic acid (BES; 10 mM final concentration) and heat-treated microcosms were also prepared. Heat-treated microcosms were treated with three cycles of autoclaving (121 °C, 20 min) and incubation at 37 °C for 17 h to germinate and subsequently destroy any spores. All microcosms were incubated at room temperature in the dark. All microcosms were prepared in triplicate and multiple sets of triplicate microcosms were prepared for each treatment to allow sacrificial sampling at different time points.

1.2.3. Methane production in microcosm headspace

Methane production in microcosm headspace was measured according to the method in Sherry et al. (2014). Briefly, methane in the headspace was sampled (100 μl) using a gas-tight syringe flushed with N_2. Methane concentrations were determined periodically by gas chromatography with flame ionization detection (GC-FID) (Model 5160, Carlo Erba, Milan, Italy), with reference to calibrations of a standard gas. The volume of methane (mmol) generated and the rate of methane production (μmol CH4 day^{-1} g^{-1} sediment/sludge) in oil-amended microcosms were statistically compared to those in the corresponding unamended (no oil) microcosms using an independent sample *t*-test as well as ANOVA with Tukey's *post hoc* honestly significant difference (HSD) (SPSS software, Table S1). The theoretical methane yield was calculated based on assumptions that 10% of the crude oil was composed of n-alkanes, of which 85% would be carbon available for conversion to methane, according to the stoichiometry $4C_{16}H_{34} + 30H_2O \rightarrow 15CO_2 + 49CH_4$ (Zengler et al., 1999).

1.2.4. Analysis of metal content of river sediment inoculum

Analysis of metals in river sediments was performed using Inductively Coupled Plasma- Mass Spectrometry (ICP-MS) by Northumbria Water Scientific Services (Wallsend, UK). The concentrations of iron, aluminium, calcium, sulphur, magnesium, sodium, potassium, lead, silicon, manganese, barium, zinc, strontium, boron, chromium, lithium, copper, nickel, arsenic, cobalt, ammonium, cadmium, mercury and selenium was determined.

1.2.5. Crude oil extraction and assessment of biodegradation

Hydrocarbon analysis was carried out on organic solvent extracts from selected microcosms. Aliquots (ca. 50 mg or less) of the solvent extracts from the microcosms were separated using the solid phase extraction method (Bennett et al., 1996) to provide a total hydrocarbon fraction. This was then separated by silver nitrate impregnated silica gel SPE chromatography into saturated and aromatic fractions (Bennett and Larter, 2000).

Gas chromatography of the saturated hydrocarbon fraction was carried out on a Hewlett Packard 5890 gas chromatograph fitted with an HP-5 coated capillary column (30 m length, 0.25 mm i.d., 0.25 μm film thickness) and a flame ionization detector. Aliquots (1.0 μl) of the samples in dichloromethane were injected into a split/splitless injector (at 280 °C) with an HP7673 autosampler. The GC oven temperature was held at 50 °C for 2 min and then increased at 4 °C/min to 300 °C where it was held for 20 min. Data acquisition and processing was done using a Thermo LabSystems Atlas chromatography data system. Linear-alkanes (nC_{12} to nC_{34}) were quantified by determining peak areas relative to the surrogate standard (squalane). Analytical reproducibility for replicate analyses (n = 4) of the C_{12}–C_{34} *n*-alkanes in the crude oil was 0.84% relative standard deviation.

To assess the degree of hydrocarbon degradation the mass of each individual *n*-alkane was summed to provide a total mass of nC_{12}-nC_{34} alkanes in triplicate oil-amended and oil-amended, heat killed control microcosms at each sacrificial time point. In addition, the ratio of nC_{17} to pristane was determined as a measure of oil biodegradation in microcosms that was independent of absolute quantification of individual alkanes. One-way ANOVA with Tukey's HSD *post hoc* test of significance was performed on nC_{12}-nC_{34} data and square-root transformed nC_{17}: pristane ratio data.

1.2.6. Microbial community analysis

1.2.6.1. DNA extraction. Microcosms were shaken to ensure homogeneity and destructively sampled at specific time points based on methane production which was used as a *proxy* for biodegradation (Table S1). Aliquots (10 ml) were removed for microbial community analysis. Sub-samples (2 ml) were also removed under N_2 from specific microcosms periodically during the incubation period (highlighted in Table S1). DNA extraction was performed on settled sediment slurry (1 ml) from all microcosms using the FastDNA Spin Kit for Soil (Q-BIOgene, California, USA).

1.2.6.2. PCR amplification of 16S rRNA gene fragments. For DGGE analysis, the variable V3 region of the bacterial 16S rRNA gene was amplified using Primer 2 and Primer 3 (~233 bp product, Muyzer et al., 1993). Touchdown PCR conditions were initial denaturation (95 °C, 3 min), followed by 24 touchdown cycles comprising denaturation (95 °C, 1min), annealing (65-53 °C; 1 min; temperature was decreased by 1 °C every second cycle) and extension (72 °C, 1 min). This was followed by 15 cycles at the lowest annealing temperature – denaturation (95 °C, 1min), annealing (53 °C, 1 min), elongation (72 °C, 1 min), and a final extension step (72 °C, 10 min). Archaeal 16S rRNA genes (~200bp product) were amplified using the primers Arch344 F-gc (Raskin et al., 1994) and Univ522 R (Amann et al., 1995) under the following conditions: initial denaturation (95 °C, 3 min) with 30 cycles of denaturation (95 °C, 1min), annealing (55 °C, 1 min) and extension (72 °C, 1 min), and a final extension step (72 °C, 10 min).

1.2.6.3. Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene fragments. DGGE analysis was conducted using a denaturing gradient gel electrophoresis system (Ingeny, Goes, The Netherlands) with a 10% acrylamide gel (Bio-Rad Laboratories Ltd, Hertfordshire, UK) formed with a 30–60% denaturing gradient for bacterial 16S rRNA gene PCR products and a 30–70% gradient for archaeal 16S rRNA gene PCR products. The 100% denaturant was 7 M urea and 40% deionised formamide. PCR products were electrophoresed for 16 h at 100 V. Gels were viewed on a Fluor-S MultiImager (Bio-Rad, Hercules, CA, USA).

1.2.6.4. Analysis of bacterial and archaeal community data. Analysis of DGGE gels was performed using the Bionumerics software package (Applied Maths, Austin, Texas, US) according to the methods of van Verseveld and Röling. (2004). Band identity and relative intensity were determined for individual community profiles. Band presence/absence data were subsequently used to calculate Bray-Curtis dissimilarity indices (Bray and Curtis, 1957) for pairwise comparisons of DGGE profiles. Bray-Curtis dissimilarity values of microbial communities from oil-amended and unamended microcosms prepared with different inocula were compared by non-metric multidimensional scaling (nMDS) analysis (Clarke and Warwick, 2001), with Primer 6 software (Version 6.1.5, PRIMER -E Ltd, UK).

1.2.6.5. DNA sequencing of excised DGGE bands. Nine dominant bands were excised from DGGE gels (4 bacterial, 5 archaeal) and DNA was eluted into sterile molecular-grade water (100 μl, Sigma) overnight before being used as template (2 μl) for PCR re-amplification using Primer 2/Primer 3 for bacterial and Arch344F/Univ522 R for archaeal 16S rRNA genes, according to the PCR conditions described above. PCR products were purified with ExoSAP-IT (GE Healthcare,

Buckinghamshire, UK). Sequencing reactions were performed on an ABI Prism DNA sequencer (3730xl Applied Biosystems, Warrington, UK) using a BigDye Terminator v3.1 Cycle Sequencing Kit (Genevision, Newcastle University, UK). Nucleotide sequences were quality assessed (Chromas version 2.3 Technelysium Pty Ltd; http://www.technelysium.com.au/chromas.html), followed by trimming of poor quality sequence read at the termini of the gene fragments (BioEdit sequence alignment editor; (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Sequences were compared against the EMBL Database, using Fasta3 (Pearson and Lipman, 1988) to identify nearest neighbours from environmental non-cultured representatives (EMBL Environmental database) and cultured representatives (EMBL Prokaryote database). To prevent large 16S rRNA databases from becoming congested due to the addition of many short 16S rRNA gene fragments we have chosen not to upload the short DNA sequences, and have instead included them in Table S5.

1.2.6.6. Quantification of 16S rRNA in oil degrading microcosms. DNA from microcosms that demonstrated a capacity for methanogenic crude oil biodegradation (River Tyne sediment, River Wear sediment, and Newton Aycliffe sludge inocula) were analysed using quantitative PCR to assess the 16S rRNA gene abundance of known *n*-alkane fermenters *(Smithella* spp.), as well as hydrogenotrophic and acetoclastic methanogens (Table S6). Gene abundances were also determined in oil-free incubations and in microcosms containing River Coquet sediment inocula, as a comparative analysis with incubations that did not exhibit oil-stimulated methane production. Log 16S rRNA gene abundance ml^{-1} from all inoculum used in the assembly of the microcosms was also conducted (Day 0). Quantification of 16S rRNA gene abundance in microcosm DNA were determined relative to standards from a 10-fold dilution series (10^8-10^1 gene copies per μl) of target DNA sequence amplified from clones. Optimal annealing temperatures for each primer pair was determined by performing a temperature gradient PCR with annealing temperatures in the range of 57 °C–70 °C (Table S6). Quantitative PCR, for all except total bacteria assays, comprised SSOFast EvaGreen Supermix (Bio-Rad Laboratories, Inc., Hercules, USA) (5 μl), PCR primers (0.25 μl of 10 pmol/μl each, Table S6), sterile water (3 μl), and DNA template (1.5 μl) made up to a final volume of 10 μl; for the total bacterial assay the mix was replaced with iQ SYBR Green Supermix (Bio-Rad). qPCR were performed in a BioRad CFX96 thermocycler and, for all assays except total bacteria, included an initial denaturation (3 min at 98 °C), followed by 40 cycles (5 s at 98 °C; 5 s at specific primer annealing temp (Table S6); for total bacteria the conditions were initial denaturation (5 min at 95 °C), 40 cycles (45 s at 95 °C, 45 s at 60.5 °C, 45 s at 72 °C).

1.3. Results

During the course of the experiment, five of the incubations showed evidence of higher rates of methane production in microcosms which received oil (oil-stimulated methanogenesis) relative to no-oil controls. These microcosms contained sediment inocula from the River Tyne, River Wear, Rydal Water, the River Tees, and sludge from an anaerobic digester from a wastewater treatment plant in Newton Aycliffe (Fig. 1; Table S1). Microcosms inoculated with sediment or sludge from the other 6 of 11 inocula (Grangemouth, Humberside, and Poland sludge, Priest Pot and Buttermere lake sediments, River Coquet sediment) did not show oil-stimulated methane production relative to the corresponding no-oil control microcosms (Fig. 1; Table S1).

Statistical comparisons of the data were initially conducted on the basis of t-tests for experimental and control pairs for each inoculum in order to select samples for subsequent hydrocarbon and microbial community analyses. However, when the full dataset was available a more rigorous statistical approach was used based on ANOVA and Tukey's *post hoc* honestly significant difference (HSD) (Fig. 1; Table S1). This approach showed that significant differences in methane production rates (and hence oil-stimulated methanogenesis) were only apparent for the River Tyne sediment and Newton Aycliffe anaerobic digester sludge. This apparent anomaly occurs because Tukey's HSD uses a single multiple comparison of means rather than several individual statistical tests (multiple pairwise t-tests) and thus the probability of erroneously determining a significant difference is lower.

Results are presented firstly for the systems which showed high levels of oil-stimulated methanogenesis (River Tyne sediment, Newton Aycliffe sludge), secondly for the systems with a potential capacity for oil-stimulated methanogenesis (River Wear and River Tees sediments, Rydal Water lake sediment) and finally the systems in which oil-stimulated methanogenesis was not observed (Grangemouth, Humberside and Poland sewage sludge and River Coquet, Priest Pot and Buttermere sediments).

1.3.1. High levels of oil-stimulated methanogenesis: River Tyne sediment and Newton Aycliffe anaerobic digester sludge

1.3.1.1. Methane production and n-alkane biodegradation. Significantly higher rates of methane production were observed in oil-amended microcosms inoculated with River Tyne sediment and Newton Aycliffe sludge compared to oil-free microcosms (Fig. 1; Table S1). Methane production rates were consistent with biodegradation of nC_{12}-nC_{34} alkanes in the River Tyne sediment microcosms and Newton Aycliffe

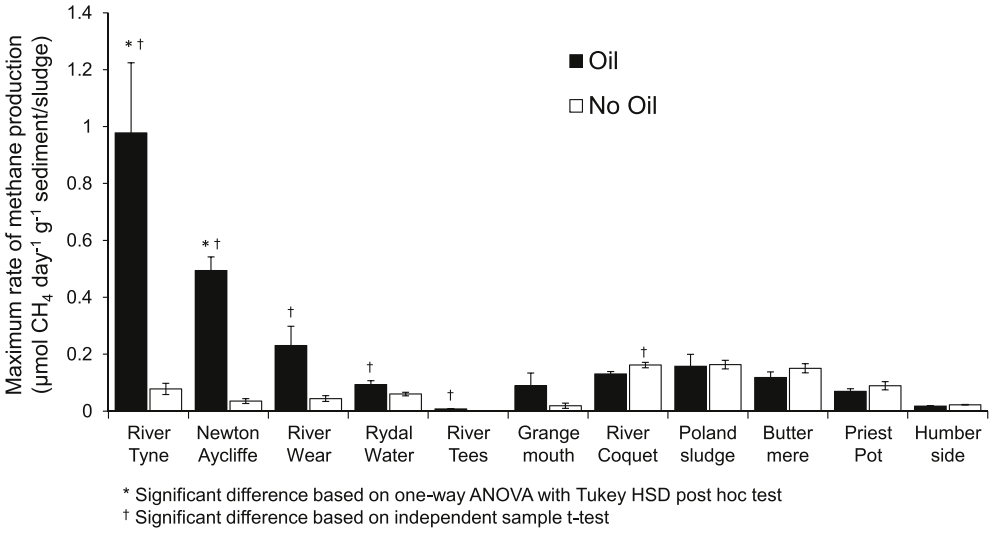

Fig. 1. Maximum rate of methane production in anaerobic oil-amended microcosms (black bars) prepared with different inocula, and control microcosms prepared without oil (white bars). Error bars are 1 x SE of triplicate microcosms. Symbols denote statistically significant differences in methane production from pairwise comparisons of oil-amended and no-oil control microcosms, based on one-way ANOVA (*) and independent sample t-tests (†). Methane production at the end of the experiment in inhibited microcosms (with bromoethanesulphonic acid (BES)) and heat-killed microcosms was negligible (BES inhibited 0.04 ± 0.01 mmol CH_4 (n = 60); heat-killed microcosms 0.01 ± 0.004 mmol CH_4 (n = 60)).

sludge microcosms amended with oil compared to autoclaved control microcosms and Day 0 microcosms (Fig. 2). In River Tyne sediment oil-amended microcosms, *n*-alkanes were significantly degraded over time, and were undetectable by Day 1150 (Fig. 2). By comparison, loss of *n*-alkanes in oil-amended, autoclaved microcosms by day 1150 was much less (Fig. 2). Similarly, in oil-amended microcosms inoculated with Newton Aycliffe sludge, *n*-alkanes were degraded significantly over time, in comparison with significantly lower n-alkane loss in oil-amended, heat-killed control microcosms (Fig. 2). Furthermore, the amount of *n*-alkanes remaining was compared against the amount of the more recalcitrant isoprenoid branched-chain alkane, pristane, as an indicator of microbial oil degradation. A significant decrease in the ratio of nC_{17}:pristane in oil-treated microcosms relative to oil added at the start of the experiment (Day 0) and autoclaved controls at the end of the incubation period, also indicated biodegradation of *n*-alkanes had occurred in the River Tyne sediment and Newton Aycliffe sludge microcosms (Table 2). The decrease in alkane concentration in killed controls (Fig. 2) likely resulted from abiotic processes, such as absorption of alkanes on the butyl rubber stoppers used to seal the microcosms, as little change in the ratio of nC_{17}:pristane was observed (Table 2).

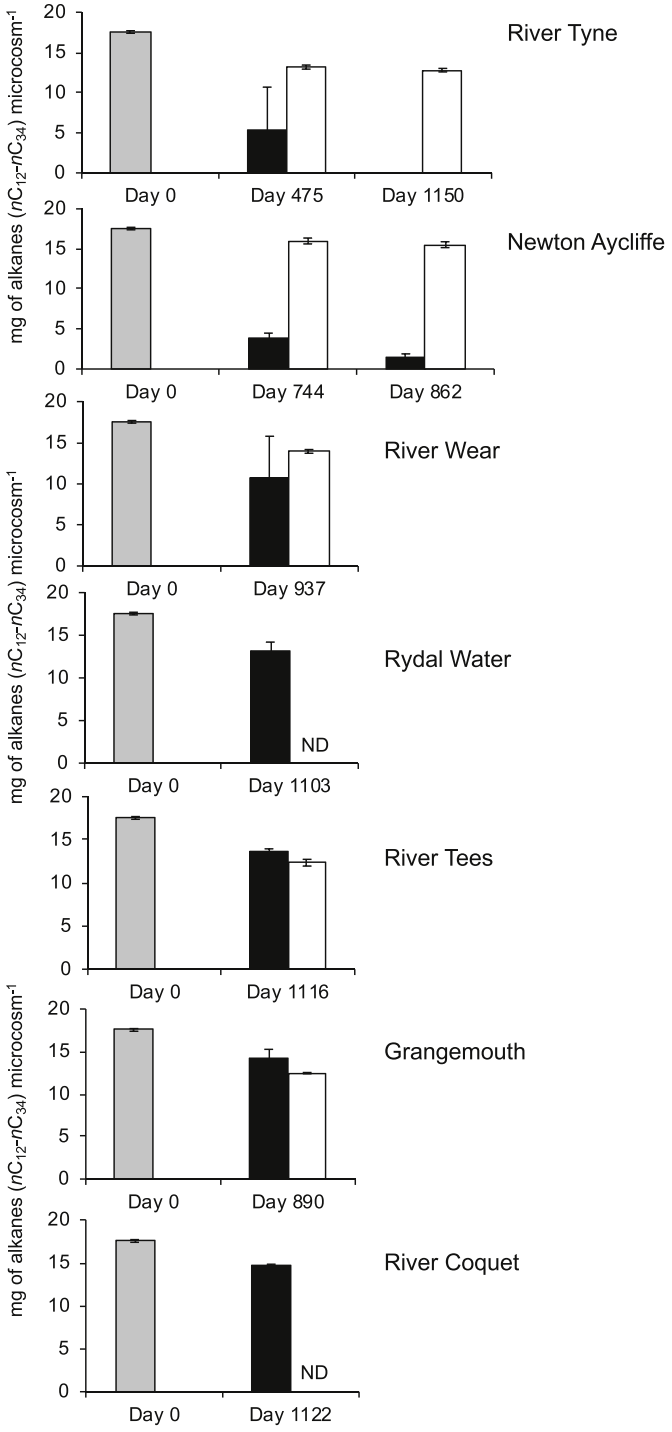

Fig. 2. Degradation of nC_{12}-nC_{34} alkanes in anaerobic oil-amended microcosms (black bars) and oil-amended, autoclaved controls (white bars) prepared with different inocula. Day 0 (grey bars) shows initial levels of nC_{12}-nC_{34} alkanes following crude oil addition to microcosms at the outset of the experiment (n = 14). ND = not determined. Error bars are 1 x SE of triplicate microcosms.

Conversion of oil alkanes to methane in oil-degrading microcosms prepared with River Tyne sediments and Newton Aycliffe sludge was confirmed by comparison of the methane yield in control microcosms that were not amended with oil. The amount of methane produced relative to no oil controls at the end of the incubation period (River Tyne Day 1150, 3.75 ± 0.36 mmol CH_4; Newton Aycliffe Day 862, 2.40 ± 0.82 mmol CH_4) was greater than the theoretical methane yield expected (River Tyne Day 1150, 0.98 ± 0.01 mmol CH_4; Newton Aycliffe Day 862, 0.77 ± 0.08 mmol CH_4) from the stoichiometric conversion of the *n*-alkanes to methane, using water as the co-reactant (Zengler et al., 1999; Anderson and Lovley, 2000). The additional methane produced during the incubations, which exceeded that predicted from the stoichiometric conversion of only the *n*-alkanes, was presumably from the degradation of other hydrocarbons in the crude oil that were not measured in our analysis.

1.3.1.2. Metals, TOC and sulfate content of river sediment inoculum. Levels of metals analysed in the River Tyne and River Tees sediments showed that River Tees sediment had elevated levels of lead, barium, strontium, lithium and boron compared to River Tyne sediment (>5-fold, >38-fold, >2-fold, >27-fold, >2-fold difference respectively; Table S2) with higher levels of total metals overall (148,717 mg/kg River Tees, 116,796 mg/kg River Tyne, Table S2).

Total organic carbon (TOC) levels in the River Tees sediment were similar to the Tyne and Wear rriver sediments investigated (TOC (%) - Tees 5.58 ± 0.22; Tyne 7.63 ± 0.03; Wear 4.31 ± 0.27; Coquet 1.37 ± 0.03).

Higher levels of sulfate were present in River Tees sediment compared to the other river sediments investigated; Tees: 0.83 ± 0.08 mM SO_4^{2-}, Coquet 0.23 ± 0.03 mM SO_4^{2-}, Wear: 0.10 ± 0.01 mM SO_4^{2-}, Tyne: 0.02 ± 0.01 mM SO_4^{2-}.

1.3.1.3. Dynamics of bacterial communities. Bray-Curtis dissimilarities were calculated from bacterial and archaeal community DGGE profiles and non-metric multidimensional scaling (nMDS) was used to assess changes in the microbial communities in microcosms inoculated from different sources. This was performed for all inocula that showed significant differences in methane production between oil-amended and oil-free control microcosms on the basis of initial pairwise comparisons using a *t*-test (sediments from River Tyne, River Wear, River Tees, Rydal Water, and sludge from Newton Aycliffe). In addition, microbial communities from microcosms inoculated with Grangemouth sludge and River Coquet sediment were chosen to represent a sludge and a sediment inoculum that did not exhibit significant oil-enhanced methanogenesis (i.e. controls) (Fig. 3, Fig. 4). Bacterial communities in oil-amended microcosms were distinct from those in control microcosms that were not treated with oil (Fig. 3A and B). There was also a clear succession over time in bacterial communities from microcosms that produced large amounts of methane from *n*-alkane biodegradation. For example, communities in replicate microcosms from methanogenic oil-degrading microcosms inoculated with Tyne sediment were similar to each other, but changes in communities occurred from day 475 to day 1150 (Fig. 3A). In microcosms inoculated with wastewater sludge, the

Table 2
Biodegradation of alkanes (nC_{12}-nC_{34}) in methanogenic microcosms as determined by nC_{17}:pristane ratio.

Location	Inoculum source	Time	nC_{17}:pristane ratio		p-value
		(Days)	Oil-amended	Heat-killed	
River Tyne	River sediment	Day 0	2.01 ± 0.03		
		Day 475	0.63 ± 0.63	1.87 ± 0.03	0.001*, 0.000†
		Day 1008	ND	ND	
		Day 1150	0.00 ± 0.00	1.90 ± 0.04	0.000*, 0.000†
River Wear	River sediment	Day 0	1.96 ± 0.02		
		Day 937	1.26 ± 0.55	1.95 ± 0.02	0.868, 0.874
River Tees	River sediment	Day 0	1.97 ± 0.03		
		Day 1116	2.01 ± 0.02	1.96 ± 0.02	1.000, 1.000
River Coquet	River sediment	Day 0	1.95 ± 0.02		
		Day 1122	1.94 ± 0.03	ND	1.000
Newton Aycliffe	Anaerobic digester sludge treating municipal waste	Day 0	1.98 ± 0.01		
		Day 744	0.42 ± 0.14	1.98 ± 0.01	0.007*, 0.028†
		Day 862	0.15 ± 0.01	1.88 ± 0.01	0.000*, 0.001†
Grangemouth	Aerobic sludge treating oil refinery waste	Day 0	ND		
		Day 890	1.92 ± 0.03	1.91 ± 0.01	1.000, 1.000
Rydal Water	Lake sediment (eutrophic)	Day 0	1.91 ± 0.02		
		Day 1103	1.83 ± 0.10	ND	1.000

*Significant difference in nC_{17}:pristane ratio between oil-amended and heat-killed microcosms.
†Significant difference in nC_{17}:pristane ratio between oil-amended and Day 0 microcosms. All comparisons were conducted at the 0.05 level using one-way ANOVA, with Tukey HSD post-hoc testing. ND; No Data.

bacterial communities in oil-amended microcosms exhibiting oil-driven methanogenesis (Newton Aycliffe sludge) were distinct from those in sludge-inoculated microcosms that did not exhibit oil-enhanced methanogenesis (Grangemouth sludge; Fig. 3B).

DNA sequencing of short 16S rRNA gene fragments from DGGE profiles in oil-amended microcosms revealed the presence of *Syntrophus* from the *Deltaproteobacteria* (96% identity), *Alkaliflexus* sp. from the phylum *Bacteroidetes* (95% identity) and *Dehalococcoidaceae* from the phylum *Chloroflexi* (100% identity) (Table S3) which were identified from a hydrocarbon-contaminated aquifer, a PAH-contaminated aquifer and produced water from a mesothermic oil field, respectively (Table S3). Members of the phylum *Firmicutes* were present in both the oil-amended and oil-free microcosms (Table S3).

1.3.1.4. Dynamics of archaeal communities. Generally, archaeal communities in oil-amended microcosms were distinct from those in control microcosms with no oil (Fig. 4). There was typically less than 40% similarity between the archaeal communities in oil-treated microcosms and their corresponding control microcosms, and greater than 60% similarity in the communities from replicate microcosms, whether or not they were treated with oil (Fig. 4). In Tyne sediment microcosms that produced large amounts of methane from *n*-alkane biodegradation archaeal communities converged, with greater than 60% similarity in the archaeal communities between day 475 and day 1008 (Fig. 4A). This

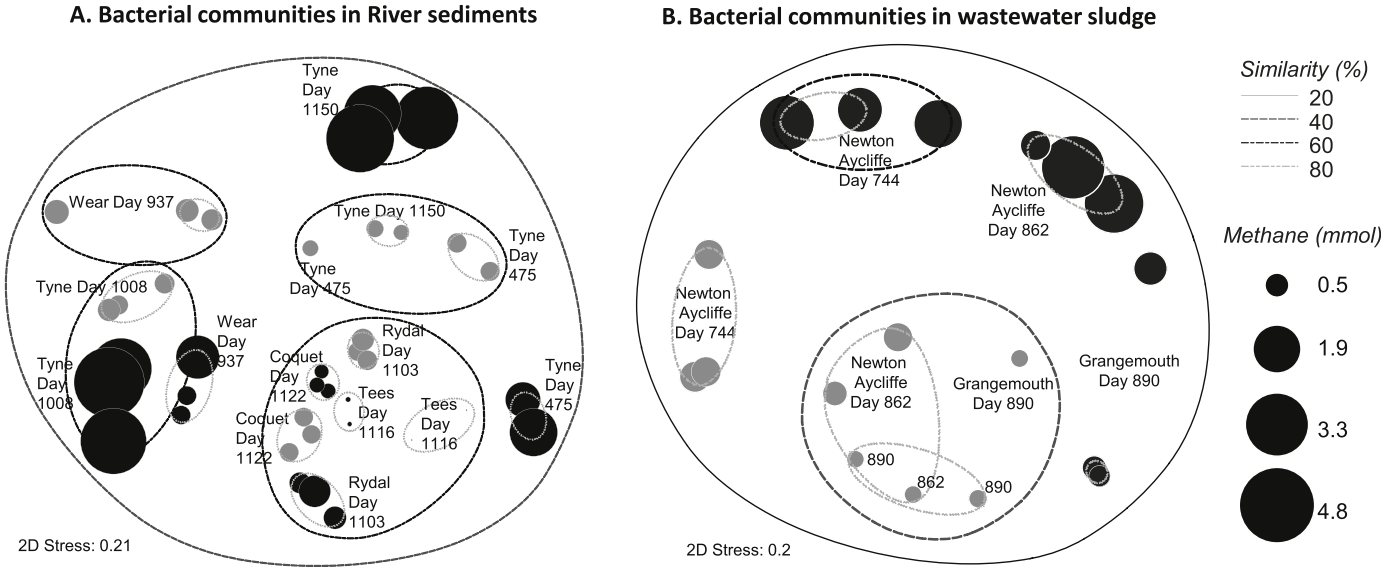

Fig. 3. Non-metric multidimensional scaling (nMDS) plots of Bray-Curtis dissimilarities of bacterial 16S rRNA gene DGGE profiles from microcosms inoculated with (A) river sediments and (B) wastewater sludge. Data from oil-amended microcosms (black circles) and from unamended control microcosms (grey circles). Total methane production (millimoles) at the time of sampling in individual microcosms is represented by the size of the symbol. No data available for Tyne Day 475 Oil replicate 1, Tyne Day 1150 No Oil replicate 3, Tees Day 1116 Oil replicate 3 and Tees Day 1116 No Oil replicate 3 because bacterial 16S rRNA PCR products were not generated in these samples.

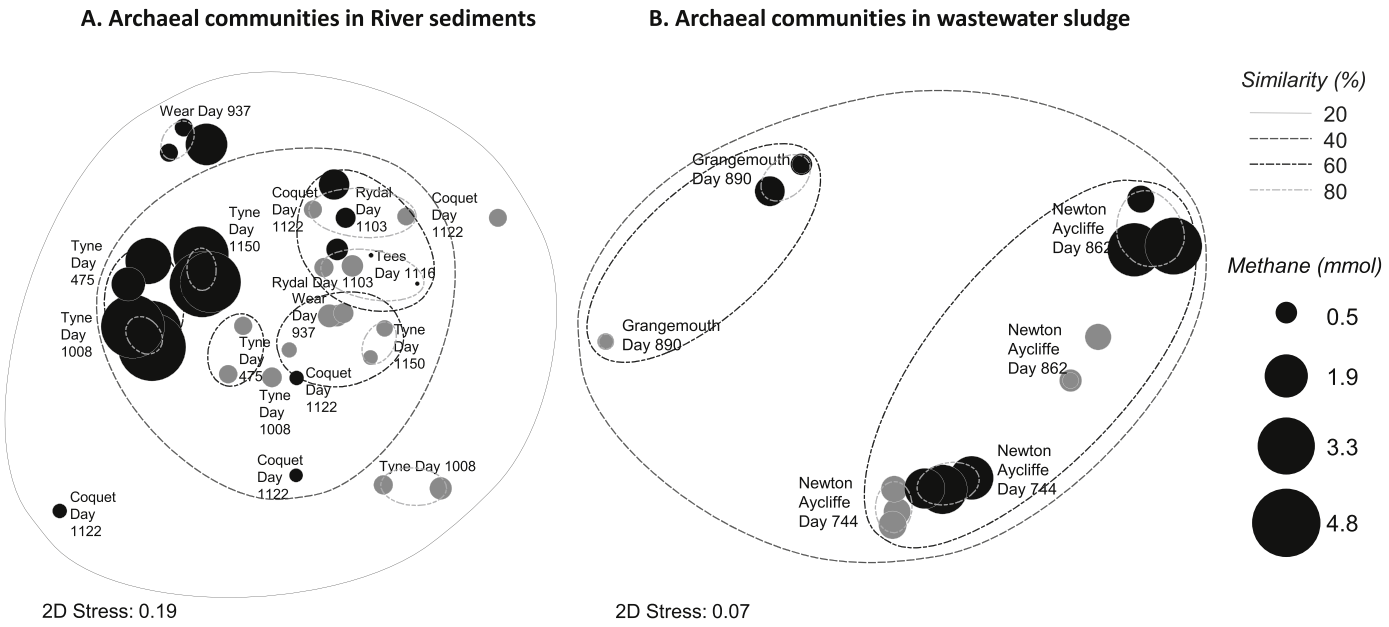

Fig. 4. Non-metric multidimensional scaling (nMDS) plots of Bray-Curtis dissimilarities of archaeal 16S rRNA gene DGGE profiles from microcosms inoculated with (A) river sediments and (B) wastewater sludge. Data from oil-amended microcosms (black circles) and from unamended control microcosms (grey circles). Total methane production (millimoles) at the time of sampling in individual microcosms is represented by the size of the symbol. No data available for Tyne Day 475 Oil replicate 1, Tyne Day 1150 No Oil replicate 3, Tees Day 1116 Oil replicate 3, Tees Day 1116 No Oil replicates 1–3 and Rydal Day 1103 No Oil replicate 1 because archaeal 16S rRNA PCR products were not generated in these samples.

contrasted with the pattern of succession seen in the bacterial communities where the communities analysed on day 475, 1008 and 1050 days were all distinct (Fig. 3A). The distinction between archaeal communities in oil-amended and oil-free microcosms was much more marked in microcosms inoculated with sludge from wastewater treatment reactors (Fig. 4B) than in sediment inoculated systems (Fig. 4A). The nMDS analysis indicated that, as with the bacterial communities, there was succession in the methanogen communities over time and a clear distinction between communities in oil-treated sludge-inoculated microcosms which showed high levels of methanogenesis from *n*-alkane biodegradation (Newton Aycliffe) compared to those that did not exhibit oil-enhanced methanogenesis (Grangemouth) (Fig. 4B).

DNA sequencing of archaeal 16S rRNA gene fragments revealed that members of the order *Methanomicrobiales* were present in the oil-amended microcosms (Table S4). Despite low sequence identities due to short fragment lengths of DNA (Table S5), *Methanocalculus* sp. (≥70%

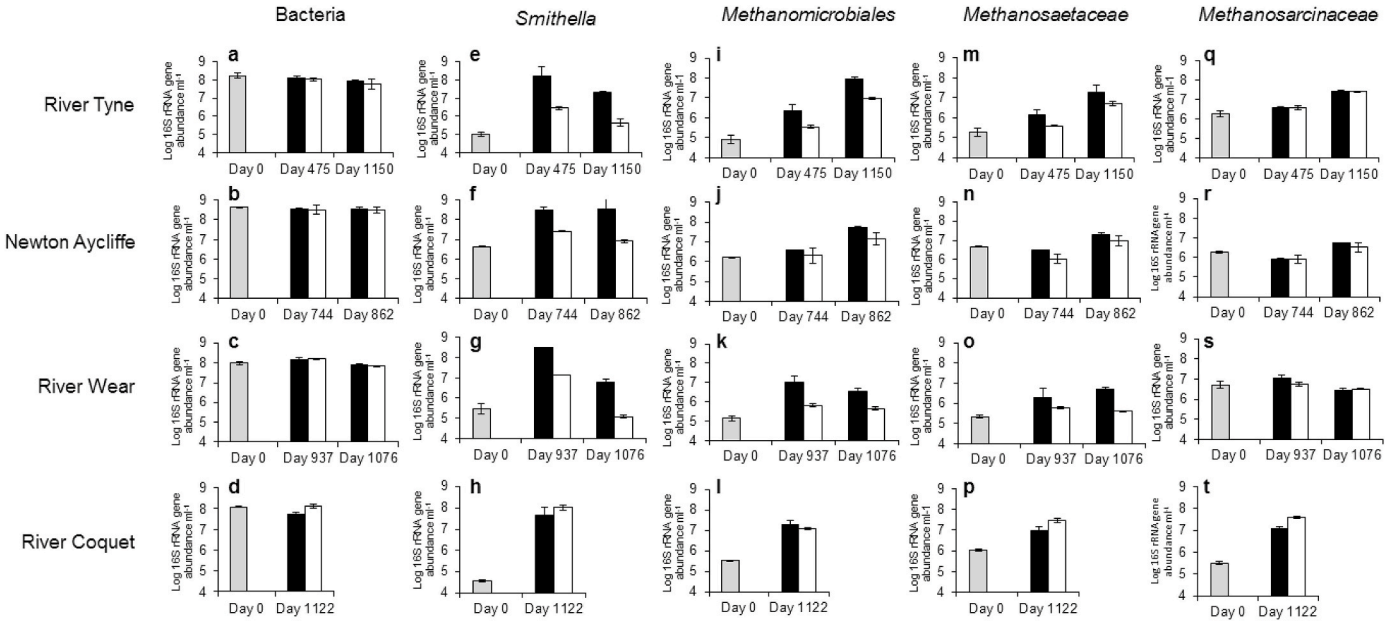

Fig. 5. Log abundance of 16S rRNA genes in methanogenic oil-degrading microcosms inoculated with sediments from the River Tyne, River Wear and sludge from Newton Aycliffe, in comparison to microcosms inoculated with sediments from the River Coquet, which did not exhibit oil-stimulated methanogenesis. Gene abundances for total bacteria, *n*-alkane fermenters (*Smithella)*, hydrogenotrophic methanogens (*Methanomicrobiales)* and acetoclastic methanogens (*Methanosaetaceae and Methanosarcinaceae)* are shown in initial sediments (Day 0; light grey bars); oil-amended incubations (black bars), and oil-free incubations (white bars). Error bars are 1 x SE of triplicate microcosms.

identity) were identified which were detected in crude-oil degrading methanogenic microcosms inoculated with River Tyne sediment (Gray et al., 2011), from methanogenic enrichment cultures prepared with sediments from a soda lake (Surakasi et al., 2007) and from leachate of a sea-based site for solid waste disposal (Mori et al., 2000) (Table S4). *Methanoculleus* sp. (≥70% identity) isolated from environments including a wetland, a landfill site and rice field soil were also identified in the oil-degrading microcosms (Table S4).

1.3.1.5. Abundance of bacterial and archaeal communities. The abundance of total bacteria, *Smithella* spp., *Methanomicrobiales*, *Methanosaetaceae and Methanosarcinaceae* was determined in microcosms containing sediments from the River Tyne and sludge from Newton Aycliffe, which showed high levels of oil-stimulated methanogenesis (Fig. 5). Comparative analysis was carried out on microcosms containing sediments from the River Wear, which showed a potential capacity for oil-stimulated methanogenesis (Section 2.2), and the River Coquet, as an example of sediments that did not exhibit the capacity for methanogenic oil biodegradation (Section 2.3).

Bacteria: Across all treatments, total bacterial gene abundances remained relatively constant during the incubation period and also between oil-amended and oil-free control microcosms (Fig. 5a-d). Generally, *Smithella* was at a similar abundance in all of the initial sources used to inoculate the microcosms, including inocula which did not go on to show oil-driven methanogenesis (Day 0, Table S7). The abundance of 16S rRNA genes from *Smithella* spp. showed more pronounced changes over time (Fig. 5e-h). In sediments from River Tyne and River Wear, and sludge from Newton Aycliffe, a marked increase in *Smithella* 16S rRNA gene abundance was observed in oil-amended incubations between Day 0 and the first time point, an increase of approx. 2–3 orders of magnitude (Fig. 5e-g). This was followed by a decrease in *Smithella* gene abundance at the later time points in microcosms containing sediments from the River Tyne (Fig. 5e) and River Wear (Fig. 5f), whereas gene abundance remained constant in microcosms containing sludge from Newton Aycliffe (Fig. 5g). Interestingly, the greatest increase in *Smithella* 16S rRNA gene abundance over the incubation period was concomitant with active periods of *n*-alkane biodegradation in sediments from the River Tyne, River Wear and in sludge from Newton Aycliffe (Fig. 2, Table 2). A similar trend in *Smithella* gene abundance was observed in oil-free incubations, but to a much lesser extent than in oil-amended incubations (Fig. 5e-g). In microcosms prepared from River Coquet sediments that did not show a capacity for methanogenic oil degradation, *Smithella* 16S rRNA gene abundance increased between Day 0 and Day 1122 in oil-amended sediments, but remained lower than in the oil-free control (Fig. 5h), and approx. an order of magnitude below that of other oil-amended microcosms (cf. Fig. 5h with Fig. 5 e, f, g).

Archaea: The abundance of the methanogens, *Methanomicrobiales*, *Methanosaetaceae and Methanosarcinaceae* was also determined. The initial abundance of methanogens in sludge from Newton Aycliffe microcosms (Fig. 5 j, n, r) was approx. 1 order of magnitude higher than the initial abundance in River Tyne sediments (Fig. 5 i, m, q), consistent with Newton Aycliffe microcosms being inoculated with anaerobic digester sludge. An increase in the gene abundance of *Methanomicrobiales* was observed in oil-amended microcosms containing sediments from River Tyne and sludge from Newton Aycliffe over the incubation period, an increase of approx. 3 and 1.5 orders of magnitude from Day 0 to the final time point, respectively (Fig. 5 I, j). Similarly, *Methanosaetaceae* increased in abundance over time in the oil-amended systems, to a greater extent than in oil-free controls (Fig. 5 m, n). The gene abundance of *Methanosarcinaceae* also increased over the time period, however there was little difference compared to the abundance in oil-free controls (Fig. 5 q, r).

In microcosms containing sediments from the River Wear, which showed a potential capacity for oil-stimulated methanogenesis, *Methanomicrobiales* and *Methanosaetaceae* were generally in lower abundance compared to sediments from the River Tyne and sludge from Newton Aycliffe (cf. Fig. 5 k, o with Fig. 5 i, m and Fig. 5 j, n, respectively). However, the abundance of *Methanomicrobiales* and *Methanosaetaceae* was appreciably enriched compared to oil-free controls (Fig. 5 k, o), even at the end of the incubation period when the abundance of *Methanomicrobiales* had decreased (Day 1076, Fig. 5k). The gene abundance of *Methanosarcinaceae* remained relatively constant throughout the time period in sediment from the River Wear, and was not noticeably enriched in oil-amended microcosms relative to oil-free controls (Fig. 5s). In microcosms prepared from River Coquet sediments that did not show a capacity for methanogenic oil degradation, the gene abundance of methanogens in oil-amended microcosms was generally similar to or below that of oil-free controls at the end of the incubation period (Day 1122, Fig. 5 l, p, t).

1.3.2. Potential capacity for oil-stimulated methanogenesis: River Wear, River Tees and Rydal Water sediment

1.3.2.1. Methane production and n-alkane biodegradation. Based on initial comparisons using a *t*-test, microcosms inoculated with River Wear, River Tees and Rydal Water sediment, showed significantly greater rates of methane generation in oil-amended microcosms compared to the corresponding no-oil control microcosms (Fig. 1). However, the rates of methanogenesis were low compared to the River Tyne and Newton Aycliffe oil-amended microcosms (Fig. 1).

In relation to these three inocula (River Wear, River Tees and Rydal Water sediment), microcosms inoculated with River Wear sediment showed the highest rates of oil-induced methanogenesis (Fig. 1). In oil-amended microcosms, the average amount of *n*-alkanes (nC_{12}-nC_{34}) appeared to decrease in abundance over time, however due to high variability in *n*-alkane data between replicate microcosms, there were no significant differences when compared to heat-killed, oil-amended control microcosms (Fig. 2). Furthermore, the nC_{17}:pristane ratio decreased over time (Table 2), however this was also not significant. Although ultimately there was no statistically significant difference in *n*-alkane biodegradation in oil-treated, oil-free and heat-treated controls, examination of data from individual replicate microcosms suggests that the capacity for methanogenic crude oil degradation was present in River Wear sediments. Two of the three oil-treated replicates showed low methane production rates and total methane production (Day 937: R2 – 0.33 mmol CH_4, R3 – 0.34 mmol CH_4) that was comparable to the no oil controls (Table S1), while the third replicate produced a much higher amount of methane (Day 937, R1 - 1.78 mmol CH_4). The high variance between biological replicates for the oil-amended River Wear microcosms most likely explains why ANOVA and Tukey's HSD indicated that methane generation in the oil-treated microcosms was not significantly higher than in no-oil controls. Interestingly, when looking at the same replicate samples individually in terms of oil chemistry and microbial community composition it was apparent that greater alkane degradation had occurred in the one replicate with high methane production (remaining nC_{12}-nC_{34} alkanes: R1; 0.57 mg, R2; 15.76 mg, R3; 15.81 mg). *Smithella* sp., implicated in syntrophic alkane oxidation, were appreciably enriched in microcosms containing River Wear sediments (Fig. 5g). These multiple lines of evidence indicate that consortia with the capacity for methanogenic oil degradation were present in one of the three randomly sampled River Wear sediment inocula. Their detection in only one of the three replicate sediment samples, however, is consistent with the organisms responsible for driving methanogenic crude oil biodegradation being present at very low abundance.

Oil-amended microcosms inoculated with lake sediment from Rydal Water showed a higher rate of methanogenesis relative to oil-free controls (Fig. 1), and microcosms inoculated with River Tees sediment showed the lowest rates of oil-induced methanogenesis (Fig. 1). Significant biodegradation of *n*-alkanes (nC_{12}-nC_{34}) was not evident in Rydal Water or River Tees sediment microcosms (Fig. 2, Table 2).

1.3.2.2. Dynamics of bacterial communities. Interestingly, the bacterial community composition in oil-treated microcosms inoculated with River Wear sediment converged with that from River Tyne (Day 1008) sediment microcosms, and was distinct from the communities in River Wear sediment oil-free control microcosms (Fig. 3A). This is consistent with the observation of oil-driven methanogenesis in some River Wear microcosms. The bacterial communities in systems which showed a low degree of methane production in oil-treated microcosms (Tees day 1116, Rydal day 1103) clustered together with communities in the non-hydrocarbon degrading controls (Coquet day 1122, Fig. 3A).

1.3.2.3. Dynamics of archaeal communities. The archaeal communities in oil-treated microcosms inoculated with River Wear sediment were distinct from those in River Tyne sediment microcosms, which showed high levels of oil-stimulated methanogenesis, and were distinct from oil-free controls as well as systems which did not show any oil-stimulated methanogenesis (Coquet day 1122, Fig. 4A). Archaeal communities in oil-treated sediment microcosms which showed very low levels of methanogenesis (Tees day 1116, Rydal day 1103) clustered together with communities from the non-hydrocarbon degrading controls (Coquet day 1122) and control microcosms that were not treated with oil (Rydal day 1103, Wear day 937, Tyne day 1150, Fig. 4A).

1.3.3. No observation of oil-stimulated methanogenesis: Grangemouth, Humberside and Poland sewage sludge and River Coquet, Priest Pot and Buttermere sediment inocula

1.3.3.1. Methane production and n-alkane biodegradation. The capacity for methanogenic oil-degradation was not detected in all environments tested; 6 out of 11 inocula showed no stimulation of methane production from the crude oil amendment (Fig. 1; Table S1). Microcosms inoculated with Grangemouth, Humberside and Poland sewage sludge and sediments from the River Coquet, Priest Pot and Buttermere did not show significant differences in methane production in oil-amended compared to oil-free control microcosms (Fig. 1; Table S1).

Microcosms inoculated with Grangemouth and River Coquet sediment did not exhibit detectable methanogenic oil-degrading capacity and were chosen as representatives of a sludge and a sediment inoculum, respectively, for comparative analysis of hydrocarbon degradation and microbial community composition. Significant biodegradation of nC$_{12}$-nC$_{34}$ alkanes, or decreases in the nC17:pristane ratio (Table 2), was not observed in these systems with respect to Day 0 or heat-killed controls (Fig. 2).

1.3.3.2. Dynamics of bacterial and archaeal communities. Bacterial and archaeal communities from the majority of oil-amended microcosms that did not show oil-driven methanogenesis (Grangemouth sludge day 890, River Coquet sediment day 1122) clustered together with communities from the control microcosms that were not treated with oil (Figs. 3 and 4).

1.4. Discussion

1.4.1. Methanogenesis from n-alkane biodegradation

The occurrence of methanogenic oil-degrading consortia in the River Tyne and, to a lesser extent, River Wear sediments may reflect their industrial heritage; both rivers run through cities that were at the heart of the industrial revolution and their sediments have been exposed to hydrocarbon pollution from coal and oil for over 100 years. The River Tyne was the UK's first major coal exporting port and the banks of both the Tyne and the Wear housed collieries, engineering works and shipyards during and beyond the industrial revolution. By contrast, rates of methanogenesis in all microcosms (with or without oil) inoculated with River Tees sediment were extremely low (Fig. 1), especially in comparison to sediments from rivers from similar industrial settings (Tyne and Wear). We therefore sought possible explanations for this apparent anomaly. Total organic carbon (TOC) levels in the River Tees sediment were similar to the Tyne and Wear River sediments investigated and thus it is unlikely that availability of organic carbon in River Tees sediment accounted for the low rates of methanogenesis observed. Higher levels of sulfate were present in the Tees sediment compared to the other river sediments investigated. However, these levels are still relatively low and we would have expected sulfate to be depleted to levels permissive for methanogenesis during the extended incubation period of these experiments (>1000 days). This is in contrast to dynamic, field situations where sulfate in river sediments may be continually replenished, and therefore the potential for sulfate-reducing bacteria to outcompete methanogens is a possibility. Levels of metals in the River Tyne and Tees sediments were analysed to determine if metal toxicity might explain the low levels of methanogenesis in the River Tees sediments. The analysis showed that River Tees sediment had elevated levels of lead, barium, strontium, lithium and boron compared to River Tyne sediment, with higher levels of total metals overall. Therefore, it is possible that metal toxicity may have restricted methanogenesis in the River Tees sediments. In the microcosms, salinity did not appear to exert a selection pressure on the methanogenic microbial communities or affect rates of methanogenesis, as the inoculum which showed the highest rates of methanogenesis were from both a brackish salinity (River Tyne sediment) and a freshwater salinity (Newton Aycliffe sludge).

The capacity for methanogenic oil-degradation was not ubiquitous in the inocula tested. Six of the inocula did not show oil-stimulated methane production even after prolonged incubation (up to 1122 days). Three of the inocula that did not exhibit oil-stimulated methanogenesis, came from relatively pristine rural environments (Priest Pot and Buttermere lake sediment and River Coquet sediment, and the remaining three inocula which did not show oil-stimulated methane production were sludges from wastewater treatment plants (Fig. 1 and Table S1; Grangemouth, Humberside, Poland). The Humberside and Grangemouth sludges were from activated sludge reactors treating wastewater from oil refineries but when incubated anaerobically, they exhibited measurable methanogenesis, a phenomenon that has been previously noted for anaerobically incubated activated sludge (Gray et al., 2002). The sludge from Poland was from an upflow anaerobic sludge blanket (UASB) reactor treating wastewater from the personal care industry which showed the highest background levels of methane initially compared to all other inocula. However, despite the addition of crude oil and continued methanogenesis in microcosms containing UASB reactor sludge, methane production in the oil-amended microcosms never exceeded that seen in the oil-free controls (Table S1). Inocula in microcosms showing no oil driven methanogenesis either did not harbour methanogenic oil-degrading consortia or they were present at abundances too low for oil-driven methanogenesis to be detected in incubations of over 1000 days. However, it is not inconceivable that oil-driven methanogenesis may have occurred in these microcosms if incubated for a longer time period.

1.4.2. Dynamics of microbial communities in methanogenic oil-degrading microcosms

Non-metric multidimensional scaling of bacterial and archaeal communities showed clear successional changes in the bacterial and archaeal communities over time in microcosms where high levels of oil-stimulated methanogenesis were measured (Tyne, Newton Aycliffe), with distinct communities in oil-amended microcosms compared to controls without oil. Similarly, in microcosms that showed a potential for oil-driven methanogenesis, the communities in oil-amended microcosms were distinct from those in microcosms without oil addition (Wear, Tees & Rydal Water).

DGGE profiles of archaeal 16S rRNA fragments showed that the majority of bands in the oil-amended microcosms migrated further through the denaturing gradient compared to those bands in the no-oil control microcosms, indicating that the DNA composition of

methanogens in oil-degrading systems potentially contained a higher content of G + C (mol %). An increase in the abundance of hydrogenotrophic methanogens (*Methanomicrobiales*) compared to acetoclastic methanogens (*Methanosaetaceae*) in oil-degrading systems is consistent with the higher mol%G + C content observed in 16S rRNA sequences recovered from microcosms in this study. DNA from the hydrogenotrophic genera, *Methanoculleus* and *Methanocalculus* detected in this study, have a mol%G + C content of 55–62 and 43–52, respectively (Boone et al., 2001). DNA from the acetoclastic genus *Methanosaeta* has a mol%G + C content of 48–54 (Patel, 2000), higher than in acetoclastic *Methanosarcina* (36–43 mol%, Boone and Mah, 2001) which was not detected in the oil-degrading systems (Table S3).

DNA extracts from microcosms that demonstrated a capacity for methanogenic crude oil biodegradation (Tyne, Wear, Newton Aycliffe) were analysed using quantitative PCR to assess the abundance of *Smithella* spp. a known *n*-alkane fermenter from the family *Syntrophaceae*, as well as hydrogenotrophic and acetoclastic methanogens. In oil degrading systems, *Smithella* abundance was orders of magnitude higher than in oil-free incubations, whereas by contrast in non oil-degrading systems *Smithella* abundance was similar to that of, or lower than, oil-free incubations. An increase in *Smithella* gene abundance in microcosms where oil-driven methanogenesis was not detected (River Coquet) may have resulted from the presence of organic matter in the sediment providing alternative substrates for *Smithella* spp. when incubated under anoxic conditions. An increase in the abundance of hydrogenotrophic methanogens (*Methanomicrobiales*) and acetoclastic methanogens (*Methanosaetaceae*) was also apparent, consistent with previous evidence that methanogenic biodegradation of hydrocarbons occurs through the concerted action of fermentative organisms together with methanogenic archaea (Gray et al., 2011; Jones et al., 2008; Mbadinga et al., 2011; Zengler et al., 1999; Scherr et al., 2012).

Bacteria from the genus *Smithella* (*Syntrophaceae, Deltaproteobacteria*) have previously been shown to be quantitatively significant in systems where methanogenic biodegradation of crude oil *n*-alkanes occurs in partnership with hydrogenotrophic methanogens (*Methanocalculus* spp. from the *Methanomicrobiales*). Complete oxidation of the crude oil alkanes occurs by syntrophic acetate oxidation to hydrogen and carbon dioxide, followed by consumption of these products by hydrogenotrophic methanogens for the generation of methane (Gray et al., 2011). A number of other studies have identified members of the *Syntrophaceae* (e.g. *Smithella* spp, *Syntrophus* spp.) as dominant organisms in methanogenic hydrocarbon impacted systems including soils, sediments, aquifers and deep coal seam-associated groundwater, suggesting that they play a widespread role in low temperature, near surface, anaerobic hydrocarbon degradation (Allen et al., 2007; Bakermans and Madsen, 2002; Dojka et al., 1998; Fowler et al., 2016; Kasai et al., 2005; Shimizu et al., 2007; Zengler et al., 1999). However, members of the *Syntrophaceae* are not always detected as the dominant fermentative organisms involved in the initial oxidation of *n*-alkanes in methanogenic hydrocarbon degradation, and previous studies have shown that members of the *Firmicutes* are most commonly detected in oil and hydrocarbon-impacted anoxic environments.

The fact that methanogen communities in oil-treated microcosms are distinct from those in oil-free microcosms, suggests that the methanogens involved in methanogenic crude oil degradation are not the same as those involved in the breakdown of indigenous organic carbon in the sediments. This is interesting given that methanogens utilise a limited range of substrates generated from organic matter degradation and these substrates are essentially the same irrespective of the nature of the organic matter from which they are derived. It may also reflect the generation of greater amounts of intermediates, such as hydrogen and acetate, during degradation of the additional organic matter provided by the oil. Alternatively, selection for distinct methanogens in oil-amended systems could reflect differential toxicity of components of the crude oil to different methanogens. In a spill from a ruptured pipeline in Bemidji, Minnesota, acetoclastic methanogens found in close proximity to the crude oil were one hundred times less abundant than hydrogen- and formate-utilizing methanogens. Furthermore, in laboratory toxicity tests the oil from the contaminated aquifer was shown to inhibit methane production from acetate, but methane was not inhibited from formate or hydrogen (Warren et al., 2003). Low molecular weight volatile hydrocarbons in a non-weathered crude oil have been shown to inhibit, but not completely stop, methanogenic alkane biodegradation (Sherry et al., 2014).

A degree of specificity in the relationship between hydrocarbon-degrading syntrophic bacteria and their methanogen partners would also explain why methanogen communities in oil-treated microcosms were distinct from those in oil-free controls. The predominant bacteria in oil-degrading microcosms were identified as *Syntrophus* (*Deltaproteobacteria)*, *Alkaliflexus* (*Bacteroidetes)* and *Dehalococcoidaceae* (*Chloroflexi),* and the archaea were hydrogenotrophic methanogens, *Methanocalculus and Methanoculleus (Methanomicrobiales),* consistent with specific syntrophic interactions inferred from a range of studies on methanogenic oil biodegradation (Gray et al., 2011; Sherry et al., 2014; Toth and Gieg, 2018). In a study of methanogenic crude oil-degrading enrichments grown on long chain fatty acids (palmitate or stearate) or alkanes (hexadecane or octadecane), it was shown that despite substrate-related changes in microbial community composition, all communities were dominated by hydrogenotrophic and acetoclastic methanogens along with *Clostridium*, *Deltaproteobacteria*, and other phyla. Furthermore, analysis of a microbial co-occurrence network revealed dense interactions amongst the syntrophic bacteria and methanogens that was maintained despite changes in the substrates for methanogenesis. The authors concluded that syntrophic interactions amongst bacteria themselves were as important as interactions between bacteria and methanogens, and that syntrophic interactions were stable over time in methanogenic enrichments (Fowler et al., 2016). By contrast, syntrophic interactions between microorganisms in oil reservoirs have recently been reviewed and appear to be broadly defined at the phylum level, rather than confined to specific phylogenetic groups (Pannekens et al., 2019). An increase in the number of studies utilizing next-generation sequencing and metagenomics for microbial community analysis of bacteria and archaea could assist in elucidating key syntrophic relationships and the metabolic roles of specific phylogenetic groups involved in methanogenic oil-degrading systems (Oberding and Gieg., 2016), and this warrants further investigation.

With regard to the ubiquity and distribution of methanogens in the natural environment, a study of Archaea in a brackish environment along the coast of the Gulf of Finland investigated fourteen sites; seven presumed to be oil-contaminated and seven presumed to be 'clean' (Yan et al., 2018). The study revealed a strong dependence of the archaeal community composition in littoral sediments, on environmental variables, particularly total organic matter, oil concentration (C10–C40), electrical conductivity, and pH (Yan et al., 2018). The impact of oil on the archaeal community composition was significant in the littoral sediment and two species of *Halobacteriaceae* (species XD46 and XDS2) were significantly more abundant in the presence of oil. Following co-occurrence network analysis of the microbial communities, which can help to reveal ecologically meaningful interactions between taxa (Freilich et al., 2010), it was shown that oil contamination stimulated distinct network structures within the archaeal communities compared to those in the 'clean' sediments. Furthermore, in a microbial survey of pristine sediments and sediments chronically impacted with polyaromatic hydrocarbons (PAH), along the coast of the Mediterranean Sea and the French Atlantic Ocean, a co-occurrence network comparison showed substantial differences in the network structure of the PAH-contaminated sediments (Jeanbille et al., 2016). It was suggested that hydrocarbon contamination may have a significant and negative effect on microbial interactions in coastal sediments rendering them more vulnerable to environmental perturbation (Jeanbille et al., 2016). In the thermodynamically challenging methanogenic degradation of hydrocarbons, microbial teamwork and taxa interactions warrant

further investigation to fully understand the mechanisms underpinning the interactions in the closely coupled syntrophic consortia.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibiod.2020.105085.

References

Allen, J.P., Atekwana, E.A., Atekwana, E.A., Duris, J.W., Werkema, D.D., Rossbach, S., 2007. The microbial community structure in petroleum-contaminated sediments corresponds to geophysical signatures. Appl. Environ. Microbiol. 73, 2860–2870. https://doi.org/10.1128/AEM.01752-06.

Ahammad, S.Z., Yakubu, A., Rodriguez, D.C., Dolfing, J., Graham, D.W., 2014. Source separation increases methane yields for waste-to-energy applications in the personal care product industry. Chem. Eng. J. 244, 195–201.

Amann, R.I., Ludwig, W., Schleifer, K.-H., 1995. Phylogenetic identification and *in situ* detection of individual cells without cultivation. Microbiol. Rev. 59 (1), 143–169.

Anderson, R.T., Lovley, D.R., 2000. Hexadecane decay by methanogenesis. Nature 404, 722–723. https://doi.org/10.1038/35008145.

Bakermans, C., Madsen, E.L., 2002. Diversity of 16S rDNA and naphthalene dioxygenase genes from coal-tar-waste-contaminated aquifer waters. Microb. Ecol. 44, 95–106. https://doi.org/10.1007/s00248-002-0005-8.

Bennett, B., Bowler, B., Larter, S.R., 1996. Determination of C0−C3 alkylphenols in crude oils and waters. Anal. Chem. 68, 3697–3702. https://doi.org/10.1021/ac960299x.

Bennett, B., Larter, S.R., 2000. Quantitative separation of aliphatic and aromatic hydrocarbons using silver ion−silica solid phase extraction. Anal. Chem. 72, 1039–1044. https://doi.org/10.1021/ac9910482.

Bennett, B., Adams, J.J., Gray, N.D., Sherry, A., Oldenburg, T.B.P., Huang, H., Larter, S. R., Head, I.M., 2013. The controls on the composition of biodegraded oils in the deep subsurface - Part 3. The impact of microorganism distribution on petroleum geochemical gradients in biodegraded petroleum reservoirs. Org. Geochem. 56, 94–105. https://doi.org/10.1016/j.orggeochem.2012.12.011.

Berdugo-Clavijo, C., Gieg, L.M., 2014. Conversion of crude oil to methane by a microbial consortium enriched from oil reservoir production waters. Front. Microbiol. 5, 197. https://doi.org/10.3389/fmicb.2014.00197.

Boone, D.R., Whitman, W.B., Koga, Y., 2001. Family II. *Methanosaetaceae* fam. nov., p 289–294. In: Boone, D.R., Castenholz, R.W. (Eds.), The Archaea and the Deeply Branching and Phototrophic Bacteria, Bergey's Manual of Systematic Bacteriology, second ed., vol. 1. Springer, New York.

Boone, D.R., Mah, R.A., 2001. Genus I. *Methanosarcina* kluyver and van Niel 1936, 400AL, emend. Mah and kuhn 1984a, 266 (nom. Cos., opinion 63 of the jud. Comm. 1986b, 492), p 269–276. In: Boone, D.R., Castenholz, R.W. (Eds.), The Archaea and the Deeply Branching and Phototrophic Bacteria, Bergey's Manual of Systematic Bacteriology, second ed., vol. 1. Springer, New York.

Bray, J.R., Curtis, J.T., 1957. An ordination of upland forest communities of southern Wisconsin. Ecol. Monogr. 27, 325–349. https://doi.org/10.2307/1942268.

Clarke, K.R., Warwick, R.M., 2001. Change in Marine Communities: An Approach to Statistical Analysis and Interpretation, Plymouth 2nd Edition. PRIMER-E, Ltd., Plymouth Marine Laboratory.

Clothier, L.N., Gieg, L.M., 2016. Anaerobic biodegradation of surrogate naphthenic acids. Water Res. 90, 156–166. https://doi.org/10.1016/j.watres.2015.12.019.

Ding, C., Ma, T.T., Hu, A., Dai, L.R., He, Q., Cheng, L., Zhang, H., 2015. Enrichment and characterization of a psychrotolerant consortium degrading crude oil alkanes under methanogenic conditions. Microb. Ecol. 70 (2), 433–444. https://doi.org/10.1007/s00248-015-0590-y.

Dojka, M.A., Hugenholtz, P., Haack, S.K., Pace, N.R., 1998. Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. Appl. Environ. Microbiol. 64, 3869–3877.

Dolfing, J., Larter, S.R., Head, I.M., 2008. Thermodynamic constraints on methanogenic crude oil biodegradation. ISME J. 2 (4), 442–452. https://doi.org/10.1038/ismej.2007.111.

Dolfing, J., Xu, A., Gray, N.D., Larter, S.R., Head, I.M., 2009. The thermodynamic landscape of methanogenic PAH degradation. Microb. Biotechnol. 2 (5), 566–574. https://doi.org/10.1111/j.1751-7915.2009.00096.x.

Embree, M., Nagarajan, H., Movahedi, N., Chitsaz, H., Zengler, K., 2014. Single-cell genome and metatranscriptome sequencing reveal metabolic interactions of an alkane-degrading methanogenic community. ISME J. 8, 757–767. https://doi.org/10.1038/ismej.2013.187.

Feisthauer, S., Seidel, M., Bombach, P., Traube, S., Knöller, K., Wange, M., Fachmann, S., Richnow, H.H., 2012. Characterization of the relationship between microbial degradation processes at a hydrocarbon contaminated site using isotopic methods. J. Contam. Hydrol. 133, 17–29. https://doi.org/10.1016/j.jconhyd.2012.03.001.

Fowler, S.J., Toth, C.R.A., Gieg, L.M., 2016. Community structure in methanogenic enrichments provides insight into syntrophic interactions in hydrocarbon-impacted environments. Front. Microbiol. 7, 562. https://doi.org/10.3389/fmicb.2016.00562.

Freilich, S., Kreimer, A., Meilijson, I., Gophna, U., Sharan, R., Ruppin, E., 2010. The large-scale organization of the bacterial network of ecological co-occurrence interactions. Nucleic Acids Res. 38, 3857–3868. https://doi.org/10.1093/nar/gkq118.

Gieg, L.M., Fowler, S.J., Berdugo-Clavijo, C., 2014. Syntrophic biodegradation of hydrocarbon contaminants. Curr. Opin. Biotechnol. 27, 21–29. https://doi.org/10.1016/j.copbio.2013.09.002.

Gieg, L.M., Davidova, I.A., Duncan, K.E., Suflita, J.M., 2010. Methanogenesis, sulfate reduction and crude oil biodegradation in hot Alaskan oilfields. Environ. Microbiol. 12, 3074–3086. https://doi.org/10.1111/j.1462-2920.2010.02282.x.

Gieg, L.M., Duncan, K.E., Suflita, J.M., 2008. Bioenergy production via microbial conversion of residual oil to natural gas. Appl. Environ. Microbiol. 74, 3022–3029. https://doi.org/10.1128/AEM.00119-08.

Gray, N.D., Sherry, A., Grant, R.J., Rowan, A.K., Hubert, C.R., Callbeck, C.M., Aitken, C. M., Jones, D.M., Adams, J.J., Larter, S.R., Head, I.M., 2011. The quantitative significance of *Syntrophaceae* and syntrophic partnerships in methanogenic degradation of crude oil alkanes. Environ. Microbiol. 13 (11), 2957–2975. https://doi.org/10.1111/j.1462-2920.2011.02570.x.

Gray, N.D., Miskin, I.P., Kornilova, O., Curtis, T.P., Head, I.M., 2002. Occurrence and activity of *Archaea* in aerated activated sludge wastewater treatment plants. Environ. Microbiol. 4 (3), 158–168. https://doi.org/10.1046/j.1462-2920.2002.00280.x.

Gray, N.D., Sherry, A., Hubert, C., Dolfing, J., Head, I.M., 2010. Methanogenic degradation of petroleum hydrocarbons in subsurface environments: remediation, heavy oil formation and energy recovery. Adv. Appl. Microbiol. 72, 137–161. https://doi.org/10.1016/S0065-2164(10)72005-0.

Gründger, F., Jiménez, N., Thielemann, T., Straaten, N., Lüders, T., Richnow, H.-H., Krüger, M., 2015. Microbial methane formation in deep aquifers of a coal-bearing sedimentary basin, Germany. Front. Microbiol. 6, 200. https://doi.org/10.3389/fmicb.2015.00200.

Jeanbille, M., Gury, J., Duran, R., Tronczynski, J., Agogué, H., Saïd, O., Ghiglione, J.-F., Auguet, J.-C., 2016. Response of core microbial consortia to chronic hydrocarbon contaminations in coastal sediment habitats. Front. Microbiol. 7, 1637. https://doi.org/10.3389/fmicb.2016.01637.

Jiménez, N., Morris, B.E.L., Cai, M., Gründger, F., Yao, J., Richnow, H.H., Krüger, M., 2012. Evidence for in situ methanogenic oil degradation in the Dagang oil field. Org. Geochem. 52, 44–54. https://doi.org/10.1016/j.orggeochem.2012.08.009.

Jones, D.M., Head, I.M., Gray, N.D., Adams, J.J., Rowan, A.K., Aitken, C.M., Bennett, B., Huang, H., Brown, A., Bowler, B.F.J., Oldenburg, T., Erdmann, M., Larter, S.R., 2008. Crude oil biodegradation via methanogenesis in subsurface petroleum reservoirs. Nature 451, 176–180. https://doi.org/10.1038/nature06484.

Kasai, Y., Takahata, Y., Hoaki, T., Watanabe, K., 2005. Physiological and molecular characterization of a microbial community established in unsaturated, petroleum-contaminated soil. Environ. Microbiol. 7, 806–818. https://doi.org/10.1111/j.1462-2920.2005.00754.x.

Li, W., Wang, L.-Y., Duan, R.-Y., Liu, J.-F., Gu, J.-D., Mu, B.-Z., 2012. Microbial community characteristics of petroleum reservoir production water amended with *n*-alkanes and incubated under nitrate-, sulfate-reducing and methanogenic conditions. Int. Biodeterior. Biodegrad. 69, 87–96. https://doi.org/10.1016/j.ibiod.2012.01.005.

Liang, B., Wang, L.-Y., Mbadinga, S.M., Liu, J.-F., Yang, S.-Z., Gu, J.-D., Mu, B.-Z., 2015. *Anaerolineaceae* and *Methanosaeta* turned to be the dominant microorganisms in alkanes-dependent methanogenic culture after long-term of incubation. Amb. Express 5, 37. https://doi.org/10.1186/s13568-015-0117-4.

Mbadinga, S.M., Wang, L.-Y., Zhou, L., Liu, J.-F., Gu, J.-D., Mu, B.-Z., 2011. Microbial communities involved in anaerobic degradation of alkanes. Int. Biodeterior. Biodegrad. 65, 1–13. https://doi.org/10.1016/j.ibiod.2010.11.009.

Mori, K., Yamamoto, H., Kamagata, Y., Hatsu, M., Takamizawa, K., 2000. *Methanocalculus pumilus* sp. nov., a heavy-metal-tolerant methanogen isolated from a waste-disposal site. Int. J. Syst. Evol. Microbiol. 50 (Pt 5), 1723–1729. https://doi.org/10.1099/00207713-50-5-1723.

Muyzer, G., De Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59 (3), 695–700.

Oberding, L., Gieg, L.M., 2016. Metagenomic analyses reveal that energy transfer gene abundances can predict the syntrophic potential of environmental microbial communities. Microorganisms 4, 5–22. https://doi.org/10.3390/microorganisms4010005.

Pannekens, M., Kroll, L., Müller, H., Mbow, F.T., Meckenstock, R.U., 2019. Oil reservoirs, an exceptional habitat for microorganisms. N. Biotech. 49, 1–9. https://doi.org/10.1016/j.nbt.2018.11.006.

Patel, G.B., 2000. Genus I. *Methanosaeta* patel and sprott (1990), 80VP, p 289–294. In: Boone, D.R., Castenholz, R.W. (Eds.), Bergey's Manual of Systematic Bacteriology, second ed., vol. 1. Springer, New York.

Pearson, W.R., Lipman, D.J., 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. U.S.A. 85, 2444–2448. https://doi.org/10.1073/pnas.85.8.2444.

Peters, K.E., Moldowan, J.M., 1993. The Biomarker Guide: Interpreting Molecular Fossils in Petroleum and Ancient Sediments. Prentice Hall, Englewood Cliffs, New Jersey, p. p363.

Raskin, L., Stromley, J.M., Rittman, B.E., Stahl, D.A., 1994. Group-specific 16S rRNA hybridisation probes to describe natural communities of methanogens. Appl. Environ. Microbiol. 60 (4), 1232–1240.

Sarkar, D., Ferguson, M., Datta, R., Birnbaum, S., 2005. Bioremediation of petroleum hydrocarbons in contaminated soils: comparison of biosolids addition, carbon supplementation, and monitored natural attenuation. Environ. Pollut. 136 (1), 187–195. https://doi.org/10.1016/j.envpol.2004.09.025.

Scherr, K.E., Lundaa, T., Klose, V., Bochmann, G., Loibner, A.P., 2012. Changes in bacterial communities from anaerobic digesters during petroleum hydrocarbon degradation. J. Biotechnol. 157 (4), 564–572. https://doi.org/10.1016/j.jbiotec.2011.09.003.

Sherry, A., Gray, N.D., Aitken, C.M., Dolfing, J., 2010. Microbial oil degradation under methanogenic conditions. In: Timmis, K.N. (Ed.), Handbook of Hydrocarbon and Lipid Microbiology. Springer, Heidelberg, Germany, pp. 3906–3917.

Sherry, A., Grant, R.J., Aitken, C.M., Jones, M., Head, I.M., Gray, N.D., 2014. Volatile hydrocarbons inhibit methanogenic crude oil degradation. Front. Microbiol. 5, 131. https://doi.org/10.3389/fmicb.2014.00131.

Shimizu, S., Akiyama, M., Naganuma, T., Fujioka, M., Nako, M., Ishijima, Y., 2007. Molecular characterization of microbial communities in deep coal seam groundwater of northern Japan. Geobiology 5, 423–433. https://doi.org/10.1111/j.1472-4669.2007.00123.x.

Siddique, T., Fedorak, P.M., Foght, J.M., 2006. Biodegradation of short-chain *n*-alkanes in oil sands tailings under methanogenic conditions. Environ. Sci. Technol. 40, 5459–5464. https://doi.org/10.1021/es060993m.

Siddique, T., Penner, T., Semple, K., Foght, J.M., 2011. Anaerobic biodegradation of longer-chain *n*-alkanes coupled to methane production in oil sands tailings. Environ. Sci. Technol. 45, 5892–5899. https://doi.org/10.1021/es200649t.

Surakasi, V.P., Wani, A.A., Shouche, Y.S., Ranade, D.R., Surakasi, V.P., Wani, A.A., Shouche, Y.S., Ranade, D.R., 2007. Phylogenetic analysis of methanogenic enrichment cultures obtained from Lonar Lake in India: isolation of *Methanocalculus* sp. and *Methanoculleus* sp. Microb. Ecol. 54 (4), 697–704. https://doi.org/10.1007/s00248-007-9228-z.

Toth, C.R.A., Gieg, L.M., 2018. Time course-dependent methanogenic crude oil biodegradation: dynamics of fumarate addition metabolites, biodegradative genes, and microbial community composition. Front. Microbiol. 8, 2610. https://doi.org/10.3389/fmicb.2017.02610.

Townsend, G.T., Prince, R.C., Suflita, J.M., 2003. Anaerobic oxidation of crude oil hydrocarbons by the resident microorganisms of a contaminated anoxic aquifer. Environ. Sci. Technol. 37, 5213–5218. https://doi.org/10.1021/es0264495.

van Verseveld, H.W., Röling, W.F.M., 2004. Cluster analysis and statistical comparison of molecular community profile data. In: Kowalchuk, G.A., de Bruijn, F.J., Head, I.M., Akkermans, A.D., van Elsas, J.D. (Eds.), Molecular Microbial Ecology Manual, second ed. Springer, New York, pp. 1373–1397.

Warren, E., Bekins, B.A., Godsy, E.M., Smith, V.K., 2003. Inhibition of acetoclastic methanogenesis in crude oil- and creosote-contaminated groundwater. Ann. Finance 7 (3–4), 139–149. https://doi.org/10.1080/10889860490465840.

Widdel, F., Bak, W., 1992. Gram-negative mesophilic sulfate-reducing bacteria. In: Balows, A., Trüper, H.G., Dworkin, M., Harder, W., Schleifer, K.-H. (Eds.), The Prokaryotes, 2nd. Edition, ume 4. Springer-Verlag, New York, pp. 3352–3379 (Chapter 183).

Yan, L., Yu, D., Hui, N., Naanuri, E., Viggor, S., Gafarov, A., Sokolov, S.L., Heinaru, A., Romantschuk, M., 2018. Distribution of Archaeal communities along the coast of the Gulf of Finland and their response to oil contamination. Front. Microbiol. 9, 15. https://doi.org/10.3389/fmicb.2018.00015.

Zengler, K., Richnow, H.H., Rossello-Mora, R., Michaelis, W., Widdel, F., 1999. Methane formation from long-chain alkanes by anaerobic microorganisms. Nature 401, 266–269. https://doi.org/10.1038/45777.

Zhou, L., Li, K.-P., Mbadinga, S.M., Yang, S.-Z., Gu, J.-D., Mu, B.-Z., 2012. Analyses of *n*-alkanes degrading community dynamics of a high-temperature methanogenic consortium enriched from production water of a petroleum reservoir by a combination of molecular techniques. Ecotoxicology 21, 1680–1691. https://doi.org/10.1007/s10646-012-0949-5.