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Dynamics and distribution of bacterial and archaeal communities in oil-contaminated temperate coastal mudflat mesocosms

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Abstract

Mudflats are ecologically important habitats that are susceptible to oil pollution, but intervention is difficult in these fine-grained sediments, and so clean-up usually relies on natural attenuation. Therefore, we investigated the impact of crude oil on the bacterial, diatom and archaeal communities within the upper parts of the diatom-dominated sediment and the biofilm that detached from the surface at high tide. Biodegradation of petroleum hydrocarbons was rapid, with a 50% decrease in concentration in the 0-2 mm section of sediment by 3 days, indicating the presence of a primed hydrocarbon-degrading community. The biggest oil-induced change was in the biofilm that detached from the sediment, with increased relative abundance of several types of diatom and of the obligately hydrocarbonoclastic *Oleibacter* sp., which constituted 5% of the pyrosequences in the oiled floating biofilm on day-3 compared to 0.6% in the non-oiled biofilm. Differences in bacterial community composition between oiled and non-oiled samples from the 0-2 mm section of sediment were only significant at days 12 to 28, and the 2-4 mm-sediment bacterial communities were not significantly affected by oil. However, specific members of the Chromatiales were detected (1% of sequences in the 2-4 mm section) only in the oiled sediment, supporting other work that implicates them in anaerobic hydrocarbon degradation. Unlike the Bacteria, the archaeal communities were not significantly affected by oil. In fact, changes in community composition over time, perhaps caused by decreased nutrient concentration and changes in grazing pressure, overshadowed the effect of oil for both Bacteria and Archaea. Many obligate hydrocarbonoclastic and generalist oil-degrading bacteria were isolated, and there was little correspondence between the isolates and the main taxa detected by pyrosequencing of sediment-extracted DNA, except for *Alcanivorax*, *Thalassolituus*, *Cycloclasticus* and *Roseobacter* spp., which were detected by both methods.

Keywords: Mudflats, Oil pollution, Archaeal communities, Hydrocarbon-degrading bacteria, Biodegradation, T-RFLP, 454 pyrosequencing

Introduction

The sediments of coastal and estuarine wetlands are highly productive and biologically diverse ecosystems that are particularly prone to anthropogenic pollution, especially petroleum hydrocarbon contamination, through industrial run-off and spills at sea (Michel et al. 2013; McGenity 2014). The low wave energy in these habitats means that oil is not readily washed back to sea and so it can persist, especially when buried in the fine-grained anoxic sediments (McGenity 2014). For example, 30 years after the *Florida* oil spill in Buzzards Bay**,** petroleum hydrocarbons remained in the coastal marsh sediments (Reddy et al. 2002). More recently, the *Deepwater Horizon* oil spill in the Gulf of Mexico spread into salt marshes, and crude oil remained in the sediments two years after the spill (Michel et al. 2013). Oil pollution disrupts marine food webs (Fleeger et al. 2003), resulting in profound effects on community composition and biogeochemical processes (Chronopoulou et al. 2013; Scott et al. 2014). The subject of this particular study is the Colne Estuary, UK, the mudflats and salt marshes of which are of scientific, social and commercial importance (www.colne-estuary.org/index.html). Despite the potential for oil to persist, Coulon et al. (2012) showed that most quantifiable oil was degraded rapidly and primarily by aerobic bacteria in the upper 1.5 cm of Colne-estuary mudflat sediments in tidal mesocosms. However, the shift in microbial community in the 1.5 cm upper sediment was modest compared with the wholesale change in <1 mm thick biofilm that detached from the surface sediment with the floating tide. Moreover, the focus was on the Bacterial (Coulon et al. 2012) and microalgae (Chronopoulou et al. 2013), whereas the archaeal community was not investigated. Also, their analysis focussed on uncultivated microbial communities, and inferences about oil-degrading activity could be made only from knowledge of previously cultivated obligate hydrocarbon degraders for which there is a strong relationship between 16S rRNA gene sequence and the ability to

degrade oil, hence the need to cultivate and characterise hydrocarbon degraders in this environment.

The Archaea represent an important component of salt-marsh and mudflat communities. At the surface their abundance is generally one to two orders of magnitude lower than that of Bacteria (McKew et al. 2011, Li et al. 2011), but at certain depths in the sediment (e.g. the sulfate-methane transition zone) they can be equally abundant (Li et al. 2011). The miscellaneous Crenarchaeota Group (MCG) represented the most abundant Archaea in estuarine sediments (Li et al. 2011), and there is evidence that they (along with MG-I crenarchaeaota) are heterotrophic (Seyler et al. 2014), potentially involved in degradation of proteins (Lloyd et al. 2013), aromatic compounds (Meng et al. 2014) and indeed a wide variety of compounds (Seyler et al. 2014). Other archaeal taxa with known function are also found in soft-grained coastal sediments, including methanogens (Munson et al. 1997), heterotrophic haloarchaea (Purdy et al. 2004) and ammonia-oxidising Archaea (Bernhard et al. 2010). Despite the growing awareness of their ecological importance, our understanding of the effects of oil pollution on sediment archaeal communities remains unclear because only a few studies have addressed this, with different outcomes (Röling et al. 2004; Taketani et al. 2010; Stauffert et al. 2014).

Given that oil mainly rests on the surface of mudflats (with some downward transport by bioturbation (Stauffert et al. 2013; 2014), and only the uppermost millimetres of tidal sediments are oxic (Miralles et al. 2007), we propose the hypothesis that bacterial as well as archaeal communities at the sediment surface (0-2 mm depth), which are in closer contact with the oil spill, will be more greatly affected than microbes at a depth of 2-4 mm, resulting in pronounced oil-induced changes in their community composition. Changes in bacterial and archaeal community composition will be a function of: (1) utilisation of hydrocarbons as a source of carbon and energy (primarily or solely by Bacteria), (2) direct inhibition by oil

toxicity (3) indirect enhancement of the growth due to reduced grazing pressure, (4) indirect changes caused by factors 1 to 3.

In this study we simulate an intertidal oil spill, characterise changes in archaeal and bacterial community composition, isolate and characterise microorganisms growing on different hydrocarbons, and measure the rate of degradation of petroleum hydrocarbons, providing an indication of the potential for natural attenuation in coastal mudflats.

Materials and Methods

Sampling site, sampling and tidal mudflat mesocosm setup

Both mudflat sediment and seawater were collected from the Pyefleet Channel (51°48'10.49"N; 0°59'25.10"E) in the Colne Estuary, UK (Figure 1a) - seawater in Jerry cans at high tide on 26 March, 2009, and 200 mudflat sediment cores (9.8 cm high and 6.5 cm in diameter) 6 days later at low tide.

The tidal mesocosms were set up in a greenhouse at the University of Essex and consisted of eight 42-litre clear polypropylene boxes (length: 52 cm, width: 44 cm, depth: 31 cm), each containing 25 cores of mudflat sediment and 10 L of seawater. Each mesocosm also had an adjoining 42-litre reservoir tank containing 30 L of seawater. One ml of weathered Forties crude oil (a medium light North Sea crude oil, weathered by distillation at 230°C for 2 h to remove the volatile fraction) was added to the surface of the cores in four of the mesocosms ("oiled" mesocosms) while the other four replicate non-oiled mesocosms served as control (Figures 1b and 1c). The weathered Forties crude oil was spiked with 0.075% w/w 1,2,3,4,5,6,7,8,9,10-decamethyl-anthracene (Sigma-Aldrich), a branched PAH that is recalcitrant to degradation and so serves as an internal marker. Submersible pumps (Weipro WH-200, 230 V) were used to propel the seawater from the reservoir tanks into the mesocosms and vice versa to simulate a 6-h tidal cycle (Figure 1b). The pumps were coupled to timers that regulated the tidal cycle; hence high tide occurred every day at 9 a.m. and 9 p.m. while low tide occurred at 3 p.m. and 3 a.m.
Evaporation of the seawater (36‰ at 2-h) was monitored by taking regular salinity measurements with a portable refractometer DIGIT-100 ATC and any salinity increase was rectified by the addition of sterile reverse-osmosis water.

Over a period of 28 days, sampling from each mesocosm (experimental quadruplicate) was carried out at 6 time points: 2 h after oil addition (t = 2-h), and then on days 3, 6, 12, 20 and 28. Complete sediment cores were sampled from the mesocosms at low tide; the surface layer of each core was finely sliced into 2 mm sections. Samples for 16S rRNA gene-based community analyses were homogenised in RNA*later*® (Ambion) and frozen at -80°C, those for petroleum hydrocarbon extraction were directly frozen at -20°C, while those for cultivation were stored at 4°C. The biofilms that had detached from the cores and were floating on the surfaces of the water at high tide were collected with the aid of sterilised spatulas, rinsed into universal bottles containing ONR7a seawater basal salts and frozen at -80°C.

Petroleum hydrocarbon analysis by GC-MS

Total petroleum hydrocarbons (TPH) were solvent extracted from oil-contaminated mudflat sediment and were analysed and quantified as described by Coulon et al. (2007).
TPH concentrations were normalised with the internal marker 1,2,3,4,5,6,7,8,9,10-decamethyl-anthracene and relative TPH abundance in each sample calculated using the equation: $\%TPH = (A_s/M_s)/(A_0/M_0) \times 100$ (Prince et al. 2003) where A_s and M_s are the concentrations of TPH and internal marker respectively in the tidal mesocosm samples, and A_0 and M_0 are the concentrations at 2-h, considered here as the initial concentration.

Isolation of bacteria from hydrocarbon enrichment cultures

Hydrocarbon enrichment cultures in liquid media were established by inoculating 200 µl of mud slurry (prepared by homogenising 1 g of mudflat sediment in 20 ml of ONR7a medium) into 125-ml serum bottles containing sterilised ONR7a medium (20 ml) (Dyksterhouse et al. 1995) with either 0.5% v/v filter-sterilised (through a 0.2 µm PTFE syringe filter) weathered

Forties crude oil, pristane, tetradecane as carbon sources. A mixture of PAHs comprising phenanthrene, pyrene and fluorene (0.167% w/v each final concentration) was also used, and prepared by dissolving them in acetone in equal proportion to a final concentration of 50 g l^{-1}, filter-sterilising and then dispensing 2 ml into serum bottles. Acetone was then allowed to evaporate leaving crystalline PAH deposits at the bottom of the bottles, followed by addition of the sediment slurry. The serum bottles were subsequently capped and crimp-sealed with PTFE-lined silicon septa.

Cyclohexane-enrichment cultures were carried out in 250 ml flasks containing 100 ml of ONR7a medium where 0.5 ml of cyclohexane was introduced through a perforated sealed glass tube, allowing diffusion of cyclohexane vapours. The flasks had hollowed septum-lined screw caps through which the glass tube containing the hydrocarbon passed.

All hydrocarbon enrichment cultures using solid media were prepared with washed agar onto which 200 μl each of the hydrocarbons and mud slurry were spread. The agar (15 g l^{-1}) was washed twice by alternately stirring with 2% acetone (on a magnetic stirrer) and rinsing in deionised water. In contrast, the PAH mixture dissolved in acetone was poured onto the surfaces of ONR7a agar plates and the acetone allowed to evaporate leaving crystalline PAH deposits. Molten agarose (3 ml per plate; 2% w/v in sterile deionised water) was poured evenly over the PAH crystals, allowed to solidify before spreading the mud slurry on the surface. Cyclohexane-enrichment cultures were prepared by placing cyclohexane-soaked GF/C filter papers on the lids of the Petri dishes and incubating in a glass desiccator jar.
All enrichment cultures were incubated at 20°C, representing the average temperature in the greenhouse; liquid cultures were also gently agitated on a platform rocker at 10 rpm.
The first round of sub-culturing from both liquid and solid cultures was carried out at week-1 and 3 to isolate bacteria involved in the different stages of hydrocarbon biodegradation.

Liquid cultures were sub-cultured by carrying out 10-fold serial dilutions and spreading 50 µl from 10^{-2} and 10^{-5} dilutions on ONR7a agar plates containing the respective hydrocarbons as described for the initial enrichments.

Nucleic acid extraction

DNA was extracted from the mudflat samples, floating biofilms and also from isolates. The full details are given in the Online Resource Material. Extracted DNA was viewed under UV light after electrophoresis in agarose gel (1% w/v in 1 × Tris-acetate-EDTA buffer at 100 V) and staining in ethidium bromide solution (0.4 µg ml^{-1}).

T-RFLP analyses of 16S rRNA genes of bacterial and archaeal communities

The 16S rRNA gene amplicons were generated by amplifying directly from DNA extracted from replicate samples of mudflat sediment and floating biofilm. PCR amplification of bacterial 16S rRNA genes was performed with primer pair FAM 63F/ HEX 1389R (Marchesi et al. 1998) (Invitrogen). PCR cycling conditions were as described by Fahy et al. (2006). Archaeal 16S rRNA genes were amplified by nested PCR using forward primer 27Fa (Fish et al. 2002) which had been FAM-labelled (Invitrogen) and universal reverse primer 1492Ru (Lane 1991) which had been HEX-labelled (Invitrogen). PCR conditions were identical to those in Fahy et al. (2006) with the exception of the hybridisation temperature that was increased to 60°C based on PCR optimisation tests. PCR products were cleaned with the QIAquick PCR purification kit (Qiagen) and ~~Cleaned PCR products (10 µl) were~~ digested (10 µl) ~~digested~~ with restriction enzymes for 3 h at 37°C; bacterial 16S rRNA gene amplicons were digested with 10 U of AluI (Fermentas) while those obtained from Archaea were digested with 10 U of Msp1(Fermentas). The remaining T-RFLP procedures were as described by Fahy et al. (2006), with slight modification. Following normalisation, fragments

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with a value representing less than 1% relative abundance were discarded. Homologous T-RFs were aligned with T-align software (Smith et al. 2005; http://inismor.ucd.ie/~talign/), and T-RF sizes less than 1 bp apart were considered the same and pooled. Replicate data were pooled by calculating mean values of the relative abundance of fragments in replicate samples; the resulting pooled data was subjected to multivariate analyses.

454 Pyrosequencing analyses of 16S rRNA genes of bacterial communities

Uncultivated microbes and specific changes in community composition of selected samples of mudflat sediment (0-2 mm oiled and non-oiled from 2-h, days 3 and 12; 2-4 mm oiled and non-oiled from day-12) and floating biofilm (day-3) were identified from 16S rRNA gene amplicon libraries generated from the extracted DNA using the 454 GS-FLX Titanium sequencing technology at the NERC Biomolecular Analysis Facility (Liverpool, UK). The full details are given in the Online Resource Material.

Sequencing of isolates

PCR targeting the 16S rRNA genes of the bacterial isolates was performed with primer pair 27F and 1492R (Lane 1991) in a Gene Amp® PCR system 9700 thermocycler (Applied Biosystems). PCR master mix and cycling conditions were as described by Fahy et al. (2006); the 16S rRNA gene amplicons were purified with the QIAquick PCR purification kit (Qiagen) and sent to GATC Biotech (http://www.gatc-biotech.com/en/index.html) for sequencing using the forward primer, 27F.

Phylogenetic analyses

Partial 16S rRNA sequences of the isolates and representative sequence from each CD-HIT cluster were compared to the NCBI database using the Basic Local Alignment Search Tool

(BLAST) for sequence similarity. Alignments of sequences were obtained with the ClustalW application (Thompson et al. 1994) of the Bioedit program. Phylogenetic trees were produced with the PHYLIP 3.695 interface (Felsenstein 2009), using the Jukes and Cantor model of nucleotide substitution, the neighbour-joining tree-building algorithm, and bootstrap resampling of 1000 data sets.

Statistical analysis

Principal Component Analysis (PCA) was carried out using XLSTAT 2014 (Addinsoft™). PCA plots of the T-RFLP community profiles of the mudflat sediment and floating biofilm were generated based on Euclidean distance metric to demonstrate the dissimilarities between community compositions of the samples. Analysis of similarities (ANOSIM) was performed with PAST (Paleontological statistics) 2.17 software package (Hammer et al. 2001) using the Bray-Curtis similarity matrix to compare the presence/absence and relative abundance of T-RFs in replicate samples. Significant hydrocarbon degradation was determined by analysis of variance (ANOVA) coupled with Tukey's HSD and Dunnett's two sided tests, also performed with XLSTAT 2014 (Addinsoft™).

Results and Discussion

Changes in hydrocarbon concentrations and other parameters in the mudflat mesocosms

The measured alkanes ranged from C_{10} to C_{39}, including branched alkanes, pristane and phytane, while the polycyclic aromatic hydrocarbons included C_0 to C_3 naphthalenes, acenaphthylene, acenaphthene, C_0 to C_2 fluorenes, C_0 to C_2 dibenzothiophenes, C_0 to C_2 phenanthrenes, fluoranthene, pyrene and chrysene, benzo[*a*]anthracene, benzo[*k*]fluoranthene, benzo[*b*]fluoranthene, dibenzo[*a*,*h*]anthracene and benzo[*g*,*h*,*i*]perylene. C_0 to C_3 corresponds to the carbon number of the alkyl group in alkylated PAHs, implying that C_0 is the native PAH with no alkyl group. The concentration of the internal marker 1,2,3,4,5,6,7,8,9,10-decamethyl-anthracene in 0-2 mm and 2-4 mm sediment sections at 2-h was 0.06 ± 0.01 µg g^{-1} sediment and 0.05 ± 0.03 µg g^{-1} sediment (mean ± SE), respectively.

Oil emulsification, an indication of biodegradation, was apparent in the oiled mesocosms by day-3. TPH rapidly decreased by about 50% (Tukey's HSD; $p<0.05$) within the first 3 days in the 0-2 mm sediment, after which no further significant TPH depletion occurred (Figure 2a; Online Resource 1). Short- and medium-chain alkanes were depleted at a faster rate than long-chain alkanes. Within this period, 61% of C_{11}-C_{16} *n*-alkanes had been depleted compared to the 35% depletion of C_{33} to C_{39} *n*-alkanes (Figure 2b). Diagnostic ratios show that the reduction in alkane concentration from 2-h to day-3 was as a result of biodegradation as there was a decrease in C_{17}/Pristane ratio from 2.45 to 1.79 and in C_{18}/Phytane ratio from 2.74 to 2.66 in the 0-2 mm sediment (where the branched alkanes are less prone to biodegradation but equally susceptible to abiotic loss). The PAHs reduced by 62% in 3 days (Figure 1c; Online Resource 1). GC-MS analyses of 0-2 mm sediment at 2-h shows that the most depleted PAH by day-3 was the semi-volatile naphthalene. Both biodegradation and volatilisation are likely to have caused this rapid decrease. In addition, the diagnostic ratio

Σchrysene/Σphenanthrene increased from 0.02 at 2-h to 0.04 at day-3 suggesting that biodegradation also contributed to the loss of PAHs.

Oil degradation was more rapid than that observed previously in similar mesocosms (Coulon et al. 2012), possibly because the oil used by Coulon et al. (2012) had been emulsified by shaking with water before addition, whereas it was not emulsified in the current study. Water-in-oil emulsions are typically difficult to degrade by microbes because of their high viscosity and low surface area (see McGenity et al. 2012). In addition, the immediate and much more rapid depletion of nutrients in the current study (compared with that of Coulon et al. (2012)) suggests that there may have been a higher starting microbial biomass in the current study, with more hydrocarbon degraders.

The 3-day decrease in TPH concentration coincided with a significant reduction (Tukey's HSD: $p<0.05$) in water-column pH (Online Resource 2c), which may be due to carboxylic acids that are by-products of hydrocarbon biodegradation (Van Hamme et al. 2003). Immediate and rapid biodegradation is suggestive of a pre-adapted microbial community, and high temperatures may have contributed to higher bacterial activity (Coulon et al. 2007) as well as volatilisation. Indeed, temperature in the greenhouse ranged between 12°C and 34.4°C and high temperatures were frequently recorded and occurred as early as day-2 of the experiment (Online Resource 2b).

Because the mesocosms were closed systems with no replenishment of nutrients, nitrate and nitrite were exhausted in 6 days in both oiled and non-oiled water columns, but significantly lower values (Tukey's HSD: $p<0.05$) at day-3 were recorded for the oiled mesocosms, which is probably due to biodegradation of hydrocarbons (Online Resource 2a).

The oiled 2-4 mm section of sediment had approximately three-fold lower TPH values than the 0-2 mm section at 2-h (Figure 2a), and there was no significant change in TPH values in

the deeper sediment (Tukey's HSD; p>0.05) throughout the study. The concentration of aromatics barely changed, possibly indicating that the PAHs were adsorbed to the sediments and not available for biodegradation.

Changes in archaeal and bacterial community composition in the mudflat sediment and floating biofilms

Circular bBiofilms that had separated from and retained the circular shape of the sediment cores, -were observed floating above the cores at high tide as also seen by Coulon *et al*. (2012). Floating biofilms were absent at 2-h and bacterial 16S rRNA gene amplicons could not be obtained from these samples on day-12. We were also unable to obtain archaeal 16S rRNA gene amplicons from the floating biofilms.

Bacterial versus archaeal mudflat sediment communities

As expected for natural samples, replicate T-RFLP profiles of oiled and non-oiled samples were quite heterogeneous (Online Resources 3 and 4), and so mean T-RFLP profiles were used for PCA visualisation. Figure 3a shows that time was the main parameter influencing bacterial community changes in the 0-2 mm mudflat sediment, and to a lesser extent, in the 2-4 mm sediment. For example, bacterial communities in oiled and non-oiled 0-2 mm sediment are similar to one another from 2-h to day-6 (Figure 3a; cluster X), but significantly differ (ANOSIM; P<0.05) from the oiled and non-oiled communities at days 12 to 28 (Figure 3a; cluster Y). Numerous factors could have contributed to these bacterial community changes, in particular the rapid decrease in dissolved nitrogen (Online Resource 2a). Oil-induced differences in the 0-2 mm sediment bacterial communities occurred much later, on days 12 and 28 (ANOSIM; P<0.05) when most of the oil had been degraded. This is contrary to other reports of an immediate shift in bacterial community composition after exposure to oil

(Bordenave et al. 2007, Païssé et al. 2010). The rapid biodegradation of oil (Figure 2a) may have negated any oil-induced toxicity towards other members of the community.

Oil had much less impact on the bacterial communities in the lower 2-4 mm sediment (ANOSIM; P = 0.1336), where TPH concentrations were lower. There are notable differences between bacterial communities at the two depths (ANOSIM; $P<0.05$) that are more noticeable from day-6 onwards (Figure 2a).

The archaeal communities in the 0-2 mm sediment layer were not significantly affected by oil (Figure 3b). This is similar to the observations of Taketani et al. (2010) where only a minor oil-induced change occurred in archaeal community structure during a 75-day tidal mangrove sediment mesocosm. In contrast, Stauffert et al. (2014) report an oil-induced reduction in the diversity of Crenarchaeota MCG in their tidal sediment microcosm study. Although Röling et al. (2004) were unable to amplify archaeal 16S rRNA genes from day 6 onwards in their laboratory microcosms of oil-contaminated beach sediments, which is indicative of a decrease in abundance, there was no clear effect of oil on archaeal communities during their field trial. The archaeal community may be collectively resistant to the toxic effects of crude oil, unlike some bacterial taxa (e.g. SAR-11; Chronopoulou et al. 2014). However, the rapid loss of hydrocarbons may also have contributed to no significant change in their composition. For the predominantly heterotrophic MCG archaea, which are abundant in marine sediments (Li et al. 2012; Stauffert et al. 2013; Meng et al. 2014) and are mostly heterotrophic (Lloyd et al. 2013; Seyler et al. 2014), and for methanogens that rely on the metabolites of other microbes, there would be no decrease in organic carbon supply from phototrophs (e.g. diatoms; Chronopoulou et al. 2013) as a consequence of oil addition. Ammonia was always detectable in the water column (Online Resource 2d), and so presumably also in the sediment, where ammonia-oxidising Archaea reside, but in low abundance relative to ammonia-oxidising bacteria in the Colne Estuary (Li et al. 2014) compared with other coastal

sediments (Bernhard et al. 2010). The lack of perturbation in organic carbon and ammonia availability are consistent with the lack of significant oil-induced change in those Archaea for which we know the phenotype. However, oil-induced changes in specific populations that were masked by the community-level analysis are a distinct possibility. We were not able to obtain archaeal 16S rRNA gene amplicons from the non-oiled 2-4 mm sediment at 2-h, day-20 and day-28, but no significant oil-induced differences in archaeal communities occurred between day 3 and 12 (ANOSIM; $P > 0.3454$).

Bacterial and diatom communities in floating biofilms compared with those at 0-2 mm depth

There were significant dissimilarities (ANOSIM; $P < 0.05$) between all oiled and non-oiled floating biofilm communities, even as early as day-3 (Figure 4). At this time diatoms made up 63% of the oiled floating biofilm sequences in contrast to 37% of the non-oiled biofilm (Figure 5), and the main changes were in the relative abundance of diatom phylotypes (Table 1). The oiled floating-biofilm communities were also distinct (ANOSIM; $P < 0.05$) from the oiled 0-2 mm sediments. There was a prevalence of diatoms and microbes belonging to the Gamma- and Alphaproteobacteria in the oiled floating biofilms compared to the oiled 0-2 mm sediment on day-3 (Figure 5; see Online Resource 98a). One oil-enriched phylotype, related to *Oleibacter,* is discussed later, and supports the finding of Coulon et al. (2012) that aerobic hydrocarbon-degrading bacteria concentrate in tidal biofilms where they are in direct contact with floating oil.

Temporal and spatial dynamics of bacterial phyla

Pyrosequencing analyses were focussed on the Bacteria, because of the minimal effects of time, oil and depth on the archaeal communities. A total of 6675 sequences were analysed; sequences of less than 440 nt constituted 7% of the total. Further details of the

pyrosequencing libraries including the distribution of sequences per sample are in Online Resource 5. A high proportion of amplicons belonged to the Bacillariophyta (diatoms) (35%), Deltaproteobacteria (18%) and the Gammaproteobacteria (12%) (Figure 5a). The very low abundance of Cyanobacteria sequences (0.6%) supports their previously reported relatively low densities in the Colne estuary (Thornton et al. 2002). Sequences with ≥ 95% identity were placed into clusters, however a diatom cluster, represented by 1639 sequences, was re-clustered using ≥ 97% sequence identity cut-off. A total of 138 clusters with ≥ 6 sequences were identified representing 54% of the total number of sequences (see Figures 6 to 8; Online Resources 6, 7 and 8).

The taxonomic classification analysis of each sample (Figure 5b) shows a dominance of diatoms at 2-h and day-3 in the 0-2 mm sediment and a slight enrichment of this group in the oiled sediments, while cyanobacterial sequences remained constantly low in all samples throughout the duration of the experiment. On day-12, however, there was a large change that corroborates the T-RFLP data shown in Figure 3a. This was largely driven by a 31% reduction in diatom relative abundance, resulting in the dominance of the Deltaproteobacteria in both oiled and non-oiled sediment. Another example of a time-associated change was the general increase of Alphaproteobacteria sequences in oiled and non-oiled 0-2 mm sediments. In contrast to these results, earlier studies (Coulon et al. 2012; Chronopoulou et al. 2013) focussing on the top 1.5 cm of Colne-estuary mudflat sediments in tidal mesocosms, reported increased abundance of both diatoms and Cyanobacteria over time in both oiled and non-oiled sediments, with Cyanobacteria dominating oiled sediments on day-21. This increase was in part ascribed to a decrease in grazing pressure by *Hediste diversicolor* and *Hydrobia* sp., due to oil toxicity (Chronopoulou et al. 2013). In the current study, by day-5, we observed the burrowing and diatom-grazing (Lopez and Levinton 1978) gastropod, *Hydrobia* sp. On day-7, 14 ± 3 (mean ± SE) individuals of *Hydrobia* were recorded per oiled

mesocosms, whereas 54 ± 13 were recorded for the non-oiled mesocosms. On day-10, *Hydrobia* numbers had increased to 38 ± 11 (mean ± SE) per oiled mesocosm. By day-27, 44 ± 8 (mean ± SE) individuals of *Hydrobia* were recorded for the oiled mesocosms, while 88 ± 18 were recorded for the non-oiled mesocosms. However, at day-12 diatom relative abundance was the same in both oiled and non-oiled surface sediments (Figure 5b), despite the previous higher abundance of *Hydrobia*. The rapid loss of oil in the current study probably allowed *Hydrobia* populations to flourish more than in the study by Chronopoulou et al. (2013), thereby explaining temporal decrease in diatoms, but other factors must explain the lack of a difference in abundance of phototrophs between oiled and non-oiled communities in the current study.

Decreased diatom abundance most likely led to reduced photosynthesis (see Chronopoulou et al. 2013) and therefore a decrease in dissolved oxygen, which possibly contributed to the dominance of the Deltaproteobacteria group on day-12. Other factors such as depletion of fixed nitrogen, the fluctuating temperature in the mesocosms (Online Resource 2), viral lysis and protozoan grazing (see McGenity et al. 2012), and accumulation of metabolic waste products may also have contributed to the time-associated changes in microbial community composition.

The variation in the mudflat communities with depth was exemplified by the largely anaerobic group, the Deltaproteobacteria, which had higher relative abundance in both the oiled and non-oiled, day-12 sediment at 2-4 mm depth compared with 0-2 mm (Online Resource ~~8b~~9b). Similarly, at day-12, sequences from the Firmicutes, predominantly members of the Clostridiales and Bacillales that are mostly anaerobes or facultative anaerobes, were more abundant in the oiled 2-4 mm layers (4%) than in the oiled 0-2 mm layers (2%).

Bacterial phyla impacted by oil

The diatoms were initially enriched by oil (Figure 5b). For instance, on day-3, they constituted 55% of the 0-2 mm oiled sediment pyrosequences, while representing 37% of the corresponding non-oiled sediment. The Chloroflexi, which consists mainly of sequences affiliated with *Anaerolineae* (85-91% sequence identity), was also slightly more abundant in the oiled sediment (data not shown). This was the only group absent in all non-oiled sediment samples; on the other hand, it constituted 0.2 % of sequences in the oiled 0-2 mm sediment at 2-h and day-3, and 1% of the oiled 2-4 mm sediment. Increased abundance of Chloroflexi in oil-rich sediments has been observed in many studies (Winderl et al. 2008; Liu et al. 2009; An et al. 2013; Sherry et al. 2013; Sutton et al. 2013; Yang et al. 2014). In particular, *Anaerolineae*, which are obligate anaerobes (Sekiguchi et al. 2003; Yamada et al. 2006) and often found in subsurface sediments (Hug et al. 2013), have been associated with anaerobic degradation of hydrocarbons. For example, *Anaerolineae* was enriched in microcosms containing oil-contaminated estuarine sediments under anaerobic and sulfate-reducing conditions (Sherry et al. 2013). Also, pyrosequence libraries of bacterial 16S rRNA genes from diesel-contaminated soil samples show a high abundance of sequences related to *Anaerolineae* compared to uncontaminated samples (Sutton et al. 2013). Hence, it is feasible that they play a role in anaerobic degradation of oil in the mudflat sediment in the mesocosms.

In contrast, members of the Bacteroidetes, many of which are specialist degraders of high molecular weight organic compounds, such as polysaccharides (Thomas et al. 2011), were negatively affected by oil (Figure 5b). For instance, Bacteroidetes sequences constituted 20% of the 2-4 mm non-oiled sediment library on day-12, while representing 8% of the corresponding oiled sediment. This is consistent with some studies that have reported the

reduction in abundance or absence of Bacteroidetes in hydrocarbon-contaminated environmental samples (Teira et al. 2007; Mortazavi et al. 2013).

Oil-impacted phylotypes

The most noteworthy phylotype enriched by oil was *Oleibacter* sp. (Cluster 1077) (Table 1; see Figure 6) with 97% sequence identity to *Oleibacter marinus* 1O18, which is an aliphatic hydrocarbon-degrading microbe (Teramoto et al. 2011). It also has 91-94% sequence identity (see Online Resource ~~9~~10) to the *Oleibacter* phylotypes detected in the Colne estuary sediment by Coulon et al. (2012). *Oleibacter* sp. constituted 5% of the pyrosequences in the oiled floating biofilm on day-3 compared to 0.6% in the non-oiled floating biofilm. Interestingly, this microbe was the only known obligate hydrocarbonoclastic bacteria (OHCB) detected in high relative abundance in this study. Other OHCB, such as *Alcanivorax*, *Thalassolituus* and *Cycloclasticus,* were present in much lower relative abundance, in clusters with fewer than six sequences; although strains belonging to these genera were cultivated (see later). Possible explanations for these findings are: (1) the low nutrient concentrations in the mesocosms (see Online Resource 2); it has been shown that the growth of most OHCB is stimulated by nutrient addition (Kasai et al. 2002a; McKew et al. 2007b; Head et al. 2006), while species of *Oleibacter* have been reported to dominate in both nutrient-supplemented and nutrient-unamended oil-polluted mesocosms (Teramoto et al. 2009); (2) the elevated and relatively constant temperatures recorded in the greenhouse may have contributed to the selection of *Oleibacter* that has been shown to play an important role in the degradation of aliphatic hydrocarbons in tropical seawater (Teramoto et al. 2009; Teramoto et al. 2011; Teramoto et al. 2013). In contrast to the current study, Coulon and colleagues (2012) did not detect *Oleibacter* in oiled floating biofilms from their tidal mesocosms, while sequences closely related to *Alcanivorax borkumensis* constituted almost 50% of the total sequences in

these biofilms. This may in part relate to the difference in sampling time, and in addition to the aforementioned differences in experimental conditions, there is likely to be a contribution from stochastic community assembly and subsequent competitive exclusion (Foster and Bell 2012).

Some members of the Alphaproteobacteria were also enriched by oil (Figure 8). Phylotypes affiliated with *Roseobacter denitrificans* NdAmb116 (cluster 1445; 99% sequence identity) were more abundant in the oiled sediment on day-12 (2.0% in 0-2 mm; 0.2% in 2-4 mm) and oiled floating biofilm (1.8%) than in corresponding non-oiled samples (Table 1). Members of the genus *Roseobacter* are metabolically diverse and also ubiquitous in coastal, and especially polluted environments (Nogales et al. 2007), forming associations with algae (Amin et al. 2012). *Roseobacter* are also frequently enriched in the presence of hydrocarbons, for example, clones of *Roseobacter* became abundant in oil-enrichment microcosms of seawater (Brakstad and Lødeng 2005; Prabagaran et al. 2007). McKew et al. (2007a) also reported the presence of 16S rRNA sequences of *Roseobacter* in both *n*-alkane and crude oil enrichments of seawater amended with nutrients. Additionally, phylotypes related to *Loktanella* ZS2-13 (cluster 1420; 99% identity) were abundant in all oiled sediment and oiled floating biofilm (Table 1).The potential of hydrocarbon degradation by *Loktanella* spp. is unclear. However, Harwati et al. (2007) isolated strains of *Loktanella* from crude oil enrichments of seawater and some of these strains could degrade *n*-alkanes between C_{10} and C_{22}. Clones of *Loktanella* have also been enriched in microcosms of diesel-amended seawater obtained in winter (Lanfranconi et al. 2010).

Diatoms (Table 1; Online Resource ~~7~~8) enriched by oil were related to *Navicula phyllepta* C15 (clusters 596_6, 596_17 and 36) with 95-99% identity with those detected by Coulon et al. (2012); and *Dickieia ulvacea* C23 (cluster 596_13), which were abundant in the day-3 oiled floating biofilm. There is evidence that diatoms, such as *Navicula*, are directly involved

in aromatic hydrocarbon degradation (See Prince 2010). However, it is more likely that they encourage growth of oil-degrading bacteria (Abed 2010) by mechanisms discussed by McGenity et al. (2012).

Oil also negatively affected some phylotypes resulting in their prevalence in non-oiled samples compared to the oiled ones (Online Resource ~~10~~11). These oil-sensitive phylotypes include *Staleya* sp. (cluster 1440; 98% sequence identity) constituting 5% in the non-oiled biofilm sequences compared to 0.3% of those from the oiled biofilm. There has been no report of the sensitivity of *Staleya* sp. to petroleum hydrocarbons.

Isolation and characterisation of oil-degrading strains

A total of 148 strains were isolated (Table 2) and partial sequencing of 16S rRNA genes from 92 isolates (selected based on colony morphology and RFLP) was carried out. Phylogenetic analysis confirmed that a large number of these isolates belong to the Gammaproteobacteria (51) (Figure 6), branching with known OHCB, such as *Alcanivorax* (28), *Thalassolituus* (4) and *Cycloclasticus* (1), as well as some generalist hydrocarbon-degrading bacteria, such as *Pseudomonas* (12), *Shewanella* (3), and *Vibrio* (2). The 35 actinobacterial isolates (Figure 7) mostly belonged to the genus *Streptomyces,* while the Alphaproteobacteria (6) (Figure 8) included *Roseobacter*- and *Thalassospira*-affiliated strains.

Two strains related to *Thalassolituus oleovorans* (99% identity) were isolated from the tetradecane-enrichment at week-1, but *Alcanivorax* spp. were not isolated from this enrichment possibly because they were out-competed by *Thalassolituus* (see McKew et al. 2007a). Furthermore, *T. oleovorans* has also been reported to have a higher growth rate and tetradecane uptake than *A. borkumensis* (Yakimov et al. 2005). In contrast, on Forties crude oil, one isolate of *Thalassolituus* was obtained compared with 13 of *Alcanivorax* species (Figure 6).

Pristane was initially used as an internal standard to estimate crude oil biodegradation in biodegradation studies as it was thought to be recalcitrant, but representatives of many genera can grow on this branched-chain alkane, including *Streptomyces*, *Microbacterium*, *Roseobacter* and *Salinibacterium* all of which to the best of our knowledge, have not been previously reported to degrade this branched-chain alkane. *Alcanivorax* isolates (with 99 – 100% identity to *A. borkumensis*) were obtained at week-1 from the pristane-enrichment. *A. borkumensis* is well known to degrade branch-chain alkanes (McKew et al. 2007a; Capello and Yakimov 2010). The cyclohexane-degrading isolates in this study consisted of a large diversity of *Streptomyces* and *Pseudomonas* phylotypes; a strain of *Pseudomonas* has been shown previously to degrade cyclohexane (Anderson et al. 1980).

Shewanella and *Pseudomonas* were the only genera with strains isolated from both alkanes and PAHs-mix enrichments. Many marine hydrocarbon-degrading microbes can only degrade one class of hydrocarbon, thus the ability to degrade multiple classes supports the view that *Shewenella* and *Pseudomonas* have an important role in hydrocarbon degradation in marine environments. Indeed, both genera were cultured from anaerobic enrichments from beach sands polluted by the Deepwater Horizon oil spill (Kostka et al. 2011). *Pseudomonas* spp. are renowned pollutant-degrading terrestrial microbes, but can also play a major role in PAH degradation in marine (Berardesco et al. 1998) and estuarine (Niepceron et al. 2010) environments. *Shewenella* spp. have been implicated in hydrocarbon degradation in diverse marine environments, including Arctic sea-ice (Deppe et al. 2005).

Isolates belonging to *Thalassospira* and one of *Cycloclasticus* were isolated on PAH plates. *Cycloclasticus* representing one of the few OHCB that specialises in PAH degradation (Dyksterhouse et al. 1995; Kasai et al. 2002b; Teira et al. 2007; Niepceron et al. 2010). *Thalassospira* spp. are also known to metabolise PAHs (Cui et al. 2008; Kodama et al. 2008)

and it was one of the dominant genera in the oil mousse formed during the Deepwater Horizon oil spill (Liu and Liu 2013).

There was little correspondence between the 454 pyrosequencing libraries and the isolated strains. For example, *Oleibacter* sp., which probably played the major role in the degradation of alkanes in the mesocosms, was not isolated from the hydrocarbon enrichments which were incubated at 20°C, a temperature lower than that needed for its optimal growth (25-30°C). However, the OHCB, *Alcanivorax*, *Cycloclasticus* and *Thalassolituus* spp. were cultured and detected in low relative abundance by pyrosequencing. Additionally, *Roseobacter* sp. was isolated from the pristane enrichment and abundantly detected in pyrosequencing libraries from the day-12 oiled sediment and oiled floating biofilm. These findings emphasise the importance of complementary approaches, whereby cultivation on hydrocarbons demonstrates the biodegradation *capabilities* of sediment communities, while 16S rRNA gene pyrosequence analysis of the community identifies those microbes that increase in relative abundance and so might be involved in oil degradation.

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Tables

Table 1 Phylogenetic clusters enriched in mudflat sediment and floating biofilm after oil addition[a]

					% of sequences in each sample									
					0-2 mm						2-4 mm		Floating Biofilm	
Cluster	**Closest Relative + Accession No.**	**% Identity**	**Order/ Family**	**Group**	2-h O +	2-h O -	d-3 O +	d-3 O -	d-12 O +	d-12 O -	d-12 O +	d-12 O -	d-3 O +	d-3 O -
1420	*Loktanella* (FJ195992.1)	99	Rhodobacteraceae	Alphap	**0.42**	0.14	**2.74**	1.27	**1.53**	0.97	**2.52**	1.62	**2.17**	1.00
1445	*Roseobacter* (FJ753033.1)	99	Rhodobacteraceae	Alphap	0.00	0.00	0.00	0.27	**2.04**	0.65	**0.21**	0.00	**1.83**	0.40
1435	*Loktanella* (FJ196061.1)	97	Rhodobacteraceae	Alphap	**0.21**	0.14	0.00	0.00	**1.02**	0.32	**0.84**	0.32	0.00	0.20
1497	*Pedomicrobium* (Y14313.1)	97	Rhizobiales	Alphap	0.00	0.28	0.09	0.18	**0.68**	0.32	**0.42**	0.16	**0.33**	0.00
1077	*Oleibacter* (AB435651.1)	97	Oceanospirillales	Gammap	0.00	0.00	0.00	0.00	**0.17**	0.00	0.00	0.00	**5.33**	0.60
596_6	*Navicula* (FJ002222.1)	98	Naviculales	Bacillariop	**4.18**	0.85	0.00	2.81	**0.51**	0.16	0.00	0.65	**12.50**	9.18
596_13	*Dickieia* (FJ002229.1)	99	Naviculales	Bacillariop	**1.04**	0.00	**2.56**	0.82	0.85	1.46	**1.26**	0.97	**5.50**	0.80
596_9	*Pennate diatom* (FJ002185.1)	98		Bacillariop	1.25	0.00	0.09	1.09	**0.51**	0.32	0.00	0.00	**2.17**	0.00
596_17	*Navicula* (FJ002222.1)	98	Naviculales	Bacillariop	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	**4.50**	0.00
36	*Diverse* (*Navicula*) (FJ002222.1)	97	Naviculales	Bacillariop	4.18	1.57	5.67	5.26	2.73	4.87	1.68	1.46	**20.33**	1.00
1287	*Navicula* (FJ002222.1)	98	Naviculales	Bacillariop	0.21	0.00	0.00	0.09	0.00	0.16	0.00	0.00	**1.67**	0.00
1107	*Cylindrotheca* (FJ002223.1)	96	Bacillariales	Bacillariop	0.00	0.14	0.00	0.00	0.00	0.00	0.00	0.00	**1.17**	0.00

[a] CD-HIT was used in generating clusters with ≥ 95% identity to known phylotypes and sub-clusters with ≥ 97% sequence identity were formed from those clusters with very large sequence set. Representative sequences from each cluster were compared to the NCBI database using the Basic Local Alignment Search Tool (BLAST) for sequence similarity. O –: non-oiled sample, Oil +: Oiled sample. All oiled samples are in shaded columns. Highlighted figures show oil enrichment of corresponding clusters.

Table 2 Summary of strains isolated from hydrocarbon-enrichments of mudflat sediment samples

	Number of Strains									
		Enrichment Time		**Carbon Source**[b]					**Media type**	
Oiled Mesocosm	**TOTAL**	Week 1	Week 3	T	F	P	C	PAH mix	Solid	Liquid
Day-12	**45**	14	31	8	4	12	15	6	19	26
Day-28	**47**	27	20	6	10	19	9	3	30	17
TOTAL	**92**	41	51	14	14	31	24	9	49	43

[b] T: tetradecane; F: weathered Forties crude oil; P: pristane; C: Cyclohexane; PAH mix: Mixture of phenanthrene, pyrene and fluorene.

Figure legends

Fig.1 Schematic diagram of the tidal mudflat mesocosms

a. Map of the Colne Estuary in Essex, UK showing the sampling site for the tidal mesocosms. **b.** Side view of a mesocosm showing the reservoir tanks and the main mesocosm tanks. At low tide, water is pumped out of the main tanks via a hose into the reservoir while the reverse occurs at high tide. **c.** Surface view showing the oiled and non-oiled mesocosms each holding 25 cores of mudflat sediment

Fig.2 Changes in Total Petroleum Hydrocarbon (TPH), alkane and PAH concentration in the mudflat sediments

a. Relative TPH abundance computed by normalising TPH values to that of internal marker 1,2,3,4,5,6,7,8,9,10-decamethyl-anthracene. The value at each point represents the mean concentration of TPH from replicate samples. Vertical bars show standard error. Values above the columns are the average internal standard (1,2,3,4,5,6,7,8,9,10-Decamethyl-Anthracene) concentration in µg g^{-1} in both 0-2 mm and 2-4 mm sediment layers at each time point. **b.** Remainder of *n*-alkanes in 0-2 mm sediment at each time point relative to 2-h. Values represent the mean amount of compounds from replicate samples. Vertical bars show standard error. Values above columns are C_{17}/Pristane and C_{18}/Phytane ratios at each time point. **c.** Remainder of PAHs in 0-2 mm at each time point relative to 2-h. Values represent the mean amount of compounds from replicate samples. Vertical bars show standard error. Values above columns are diagnostic ratios at each time point. Naphthalenes: C_0-C_3 naphthalene; Fluorenes: C_0-C_2 fluorene; Dibenzothiophenes: C_0-C_2 dibenzothiophene; Phenanthrenes: C_0-C_2 phenanthrene; BaA: Benzo[*a*]anthracene; BkF: Benzo[*k*]fluoranthene; BbF: Benzo[*b*]fluoranthene; diBahA: Dibenzo[*a,h*]anthracene; BghiP: Benzo[*g,h,i*]perylene.

Fig.3 Changes in microbial community composition of mudflat sediments

a. PCA plot of mudflat sediment bacterial communities represented by the first two (F1 and F2) and first and third (F1 and F3) principal component factors showing 41% and 34% variability respectively between samples. Cluster X: oiled and non-oiled 2-h to day-6 sediment; Cluster Y: oiled and non-oiled day-12 to day-28 sediment. **b.** PCA plot of mudflat sediment archaeal communities represented by the first two (F1 and F2) and first and third (F1 and F3) principal components. PCA was based on Euclidean distance.

Fig.4 Changes in bacterial community composition of 0-2 mm sediment and floating biofilms

The PCA plot is represented by the first two (F1 and F2) and first and third (F1 and F3) principal components. PCA was based on Euclidean distance.

Fig.5 Taxonomic classification of 16S rRNA gene 454 pyrosequencing reads from selected mudflat sediment and floating biofilm samples

Sequences were classified on Greengenes using the RDP and NCBI classifier. The unassigned group contains sequences with no blast hits. **a.** Proportion of reads per higher taxa. **b.** Taxonomic composition of mudflat sediment and floating biofilm samples. Oil –ve: non-oiled sample, Oil +ve: Oiled sample. Number of reads per

sample: 2-h oiled: 479 sequences, 2-h non-oiled: 702 sequences, day-3 oiled: 1093 sequences, day-3 non-oiled: 1103 sequences, 0-2 mm day-12 oiled: 587 sequences, 0-2 mm day-12 non-oiled: 616 sequences, 2-4 mm day-12 oiled: 476 sequences, 2-4 mm day 12 non-oiled: 618 sequences, biofilm oiled: 600 sequences, biofilm non-oiled: 501sequences

Fig.6 Phylogenetic tree based on partial 16S rRNA (~395 bp) gene sequences of mudflat isolates and CD-HIT-generated clusters with ≥ 6 sequences (from the 454 sequencing libraries) belonging to the Gammaproteobacteria

Sequences were aligned with the ClustalW application on the Bioedit programme and the tree was constructed with the Phylip suite using DNAdistance followed by the Neighbour-joining tree-making programme. Bootsrap values were generated from 1000 data sets. Branches with an asterisk are supported by bootstrap values ≥70%. The bar represents the average nucleotide substitution per base. The 16S rRNA gene of *Haloferax volcanii* NCIMB 2012 (AY425724.1) was used as outgroup.

† Clusters containing phylotypes that are also present in day-12 oiled mudflat sediment

¶ Clusters containing phylotypes that were negatively affected by oil addition

Fig.7 Phylogenetic tree based on partial 16S rRNA (~430 bp) gene sequences of mudflat isolates and CD-HIT-generated clusters with ≥ 6 sequences (from the 454 sequencing libraries) belonging to the Actinobacteria

Sequences were aligned with the ClustalW application on the Bioedit programme and the tree was constructed with the Phylip suite using DNAdistance followed by the Neighbour-joining tree-making programme. Bootsrap values were generated from 1000 data sets. Branches with an asterisk are supported by bootstrap values ≥70%. The bar represents the average nucleotide substitution per base. The 16S rRNA gene of *Haloferax volcanii* NCIMB 2012 (AY425724.1) was used as outgroup.

† Clusters containing phylotypes that are also present in day-12 oiled mudflat sediment

Fig.8 Phylogenetic tree based on partial 16S rRNA (~450 bp) gene sequences of mudflat isolates and CD-HIT generated clusters with ≥ 6 sequences (from the 454 sequencing libraries) belonging to the Alphaproteobacteria

Sequences were aligned with the ClustalW application on the Bioedit programme and the tree was constructed with the Phylip suite using DNAdistance followed by the Neighbour-joining tree-making programme. Bootsrap values were generated from 1000 data sets. Branches with an asterisk are supported by bootstrap values ≥70%. The bar represents the average nucleotide substitution per base. The 16S rRNA gene of *Haloferax volcanii* NCIMB 2012 (AY425724.1) was used as outgroup.

† Clusters containing phylotypes that are also present in day-12 oiled mudflat sediment

§ Clusters containing phylotypes that were enriched by oil addition

¶ Clusters containing phylotypes that were negatively affected by oil addition