

**Hydrocarbon biodegradation in coastal mudflats: the central role of dynamic tidal
biofilms dominated by aerobic hydrocarbonoclastic bacteria and diatoms**

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ABSTRACT

Mudflats and salt marshes are habitats at the interface of aquatic and terrestrial systems that provide valuable ecosystem services. Therefore, it is important to determine how catastrophic incidents, such as oil spills, influence the sediment microbial communities that are pivotal to ecosystem function, and to identify the oil-degrading microbes that mitigate ecosystem damage. In this study, an oil spill was simulated using a tidal chamber containing intact diatom-dominated sediment cores from a temperate mudflat. Changes in the composition of Bacteria and diatoms from both the sediment and tidal biofilms that had detached from the sediment surface, were followed as a function of hydrocarbon removal. The hydrocarbon concentration in the upper 1.5 cm of sediments decreased by 78% over 21 days, with at least 60% attributed to biodegradation. Most phylotypes were minimally perturbed by oil addition, but at day 21, there was a ten-fold increase in cyanobacteria in the oiled sediment. Throughout the experiment, phylotypes associated with aerobic degradation of hydrocarbons, including PAHs (*Cycloclasticus*) and alkanes (*Alcanivorax*, *Oleibacter*, *Oceanospirillales* strain ME113), substantively increased in oiled mesocosms, collectively representing 2% of the pyrosequences in the oiled sediments at day 21. Tidal biofilms from oiled cores however, at day 22 consisted mostly of phylotypes related to *Alcanivorax borkumensis* (49% of clones), *Oceanospirillales* ME113 (11% of clones) and diatoms (14% of clones). Thus, aerobic hydrocarbon biodegradation is most likely to be the main mechanism of crude-oil attenuation in the early weeks of an oil spill, with tidal biofilms representing zones of high hydrocarbon-degrading activity.

INTRODUCTION

The explosion of Deepwater Horizon in the Gulf of Mexico released about 700 million litres of crude oil (7), resulting in large-scale public concern, particularly as the oil slick approached the shoreline. Such catastrophic incidents and numerous smaller spills remain a serious risk to marine ecosystems, especially vulnerable coastal zones, including highly productive estuaries, mudflats and salt marshes. In contrast to incidents of oil pollution on land, which are relatively contained and predictable in consequence, those occurring in coastal marshes are difficult to control (13, 48), and remediation by physical removal of oil is often impractical in muddy sediments. Oil concentrates on the surface of mudflats because of the presence of a thin mobile surface layer of water-saturated mud and extracellular polymeric substances (EPS) that is largely impermeable to oil, although oil can penetrate through holes made by burrowing animals (19). If spilled crude oil is not rapidly degraded at the surface the likelihood of burial increases; and in clay-rich intertidal mudflats a combination of anoxia and sorption to sediment particles greatly reduces the potential for biodegradation, especially for high molecular weight polycyclic aromatic hydrocarbons (PAHs). Thus, some of the most toxic components of oil can remain in coastal sediments for decades, and are released much later into the environment by burrowing animals, erosion and dredging (48). Our understanding of the particular microbes involved in oil biodegradation and their interactions remains limited, and there is a pressing need to understand how microbial mats respond to crude oil pollution, and to develop knowledge-based strategies in order to promote ecological repair.

The aim of this study was to follow the loss of hydrocarbons in relation to changes in microbial community in mudflat sediment mesocosms immediately after an experimental spill, without nutrient amendment. The stratified nature of the microbial communities and the surface biofilms were maintained in intact sediment cores, with simulated tidal cycles. The

Colne Estuary, investigated here (Fig. 1), consists of mudflat and salt-marsh habitats that are recognised internationally for their importance to the conservation of biodiversity and for their oyster fisheries (www.colne-estuary.org/Index.html). However, the estuary lies near to one of the busiest shipping lanes in the world, and so is susceptible to oil pollution. Owing to the high microbial diversity (30), relatively high nutrient concentrations (23) and previous exposure to hydrocarbons by boat traffic, our hypotheses were that biodegradation would be rapid and that these intertidal sediments would house a wide diversity of oil-degrading microbes. However, because petroleum hydrocarbons may represent a small fraction of the total organic carbon pool in mudflat sediments (50), it is often difficult to associate subtle changes in the sediment microbial community with crude-oil addition, especially when using intact sediment cores. In order to address this, we used 454 pyrosequencing to identify putative hydrocarbon-degrading bacteria in the upper 1.5 cm of the sediment, and also analysed those bacteria in most intimate contact with the oil, i.e. living on a thin biofilm that detached from the sediment surface at high tide. The dominance of aerobic hydrocarbonoclastic bacteria on these tidal biofilms suggests that they are hot spots of hydrocarbon degradation in coastal wetlands that could serve to transport primed hydrocarbon-degrading communities to other parts of the marsh and further afield.

MATERIALS AND METHODS

Field site, sampling and tidal mud-flat mesocosms set-up

Sediment samples were obtained from mudflats at the mouth of the Colne Estuary, UK (Fig. 1). Sediment cores (9.8 cm high, 6.5 cm diameter), designated 'CL' (Colne Live) and 'COL' (Colne Oil Live), were collected on 3 July 2006 at low tide. Three days earlier, sediment was collected and then autoclaved for 30 and 80 min on consecutive days (designated 'COK', Colne Oil Killed). Surface seawater was collected at high tide from the same part of the

estuary, stored at 4°C for a maximum of 6 days before use. Seawater added to the autoclaved cores was filtered through a 0.2 µm pore-size filter and autoclaved for 20 min.

The mesocosm experiments were located in an outdoor plot at the University of Essex. Nine split-plot randomized, independent mesocosm units consisted of 42 × 32 × 25.5 cm (l × w × h) transparent polypropylene containers that held 20 randomized sediment cores, and had a tube fitted through a hole 8.5 cm from the top to allow exchange of water (Fig. 2a). The reservoir (45 × 22 × 24 cm) in each unit was topped up with seawater to replace sampled water. Submersible pumps (Little Giant, 115 V) were used to drive the flow of water (Fig. 2b). A 6 h pumping cycle was established to simulate the natural tides. For consistency, tides were set to occur at the same time every day, and tidal flux took about 1 h from the initiation of the ebb or flood tide. In order to prevent dilution by rainfall and minimise evaporative loss, the mesocosms were covered with a light-transparent Plexiglas sheet under which solar light sensors and automated air temperature probes were fixed. The mesocosm CL1 was further equipped with three automated temperature probes (Fig. 2a) in order to monitor water and sediment temperature variation during the experiment (Fig. S1).

Forties blend crude oil (North Sea, UK) was distilled at 230°C to remove the volatile fraction. Then it was vigorously mixed with sterile seawater at a ratio of 25% v/v by shaking manually for a few minutes until an emulsion was obtained, of which 4 ml was added to each core, corresponding to an oil loading of 300 ml m⁻². Triplicate mesocosms were established for the following treatment and controls: oil-contaminated (COL-1, 2 and 3), uncontaminated (CL-1, 2, and 3), and killed oil-contaminated (COK-1, 2 and 3).

In order to allow time for the oil to interact with the sediment, the cores were kept at low tide until the next afternoon, when the tide was initiated and the first samples were taken on the morning of 5 July 2006 (designated as day 0). A single sediment core from each mesocosm (giving experimental triplicates) was taken in the morning of days 2, 5, 8, 12, 16

and 21. The upper 1.5 cm of each core was sectioned, homogenised and split into three subsamples, and frozen at -80°C for hydrocarbon extraction and nucleic acid analysis.

Petroleum hydrocarbon analysis in mesocosm sediment and seawater

Sediment samples (2 g) were chemically dried with 2 g of anhydrous sodium sulphate. Dried samples were extracted with 6 mL of hexane:dichloromethane (1:1) by horizontal shaking at 150 oscillations per min over 16 h and finally sonicated for 30 min at 20°C. After centrifugation (5897 *g* for 20 min), extracts were cleaned on solid phase extraction (SPE) cartridges as recommended by the manufacturer (SPE Supelclean Envi™-18, Supelco Bellefonte, USA). Extracts were diluted 10 times for gas chromatography mass spectrometry (GC-MS) analysis. Seawater samples (40 ml) were treated as described by Coulon et al. (6), except that elution from the SPE cartridges with 5 mL of hexane:dichloromethane (1:1) was followed by evaporation to 0.7 mL in an ice bath prior to GC-MS analysis.

Deuterated internal standards and quantification by GC-MS analysis has been described previously (6). Hydrocarbon analytes from crude oil were normalized to 17 α (H), 21 β (H) C₃₀-hopane naturally present in crude oil and the percent depletion of analytes was calculated as described by Venosa et al. (47). Use of surrogate solutions of ASTM D5442 standard (C₁₂-C₆₀) and Quebec Ministry Environment PAH mix (6) demonstrated that variation in the reproducibility of extraction and quantification of six sediment and water samples was +/- 10% and 8%, respectively.

Bacterial community analysis: general approach

Changes in the composition of Bacteria inhabiting the sediment were investigated by DGGE using amplified cDNA from reverse-transcribed 16S rRNA, and by two approaches using directly amplified 16S rRNA genes: terminal restriction fragment length polymorphism (T-

RFLP) analysis and 454 pyrosequencing. Floating, circular biofilms that had detached from sediment cores with the tide (tidal biofilms) were collected on day 22 with a spatula, and frozen immediately for analysis of the bacterial community by T-RFLP, cloning and sequencing. These biofilms remained intact when sampled and had a mucoid consistency.

DGGE analysis of reverse-transcribed bacterial 16S rRNA from the upper 1.5 cm sediment

Nucleic acids were extracted from 0.5 g of sediment as described by McKew et al. (28) except that the polyvinyl-polypyrrolidone step was not used. RNA was obtained by DNase digestion (28) and cDNA was synthesized by reverse transcription of the isolated RNA following Bioline's BioScript™ protocol. Total RNA (1 µl) was used in a 20 µl reaction which contained 10 pmol of reverse primer (5'-ATT ACC GCG GCT GCT GG-3'), 40 mM dNTP mixture and 4 µl of reaction buffer (Bioline). Reverse transcription was performed using a GeneAmp® PCR system 9700 Thermocycler (Applied Biosystems) at 70°C for 5 min, 40°C for 60 min and 70°C for 10 min. In order to amplify the V3 region of the 16S rRNA from Bacteria (which corresponds to positions 341 to 534 in *Escherichia coli*), 1 µl of the cDNA was used in a PCR with primers and conditions described by McKew et al. (28). The DGGE protocol (28, 31) employed a denaturing gradient from 45% to 60%, and the gel was stained using silver nitrate solution 0.1% (w/v) for 20 min. It was then fixed (10% (v/v) ethanol and 0.5% (v/v) glacial acetic acid) for 30 min, developed (0.15% (w/v) NaOH and 0.8% (v/v) formaldehyde), transferred to the fixing solution for 15 min and rinsed with distilled water. Bands were excised and incubated in 60 µl of water at 37°C overnight, then stored at 4°C until analysis. Re-amplification, cleaning, sequencing and analysis were as described previously (28). Those bands containing mixed sequences were amplified and cloned into pCR2.1 vector using a TA cloning kit version V (Invitrogen), following the

manufacturer's instructions. Sequence identities were compared with the GenBank nucleotide database library by BLAST on-line searches (3).

Pyrosequencing analysis of 16S rRNA genes from the upper 1.5 cm of the sediments

DNA from the extraction described above was used to examine the bacterial community in CL and COL samples from days 2, 12 and 21. PCR of bacterial 16S rRNA genes (V3 region), barcoding, pooling, cleaning, quantifying and pyrosequencing were performed as described by McKew et al. (30). Barcodes and the forward primer sequence were removed and only sequences of at least 133 bp were processed. Sequences were classified using the Greengenes 16S rRNA gene database (10), and based on a NAST (Nearest Alignment Space Termination) alignment (9). Broad taxonomic groupings were based on the RDP classification. Phylogenies were obtained as described previously (30) and Principal Component Analysis (PCA) was performed using the UniFrac web application (27). Cluster analysis was performed using a modified Mothur pipeline (41). CD-Hit (26) was used to cluster sequences that were $\geq 95\%$ similar. Only clusters with ≥ 6 sequences were analysed further. The representative sequence from each cluster was subjected to BLAST analysis (3).

Terminal restriction fragment length polymorphism (T-RFLP) analysis, cloning and sequence analysis of 16S rRNA genes from the tidal biofilms

Circular biofilms floating directly above their corresponding core were sampled from triplicate CL and COL mesocosms on day 22, and subjected to DNA extraction using the method described above. PCR of 16S rRNA genes and T-RFLP methods were as described by Fahy et al. (15). In order to identify the main bacteria in the tidal biofilms, PCR products obtained with bacterial primers 27F (5'-AGA GTT TGATCM TGG CTC AG-3') and 1492R (5'-TAC GGY TAC CTT GTTACG ACT T-3') were cloned and partially sequenced.

Duplicate PCR products were pooled and cleaned with Qiagen PCR purification kits. Cloning was carried out using the TOPO TA Cloning Kit (Invitrogen), as per manufacturer's instructions. Inserts were amplified with primers M13F and M13R and grouped by RFLP with CfoI and AluI. Clones with identical RFLP patterns for both enzymes were assumed to belong to the same phylogenetic group, and at least one representative was sequenced. Sequencing reactions with primer 518R (5'-CGT ATT ACC GCC GCT GCT GG-3') and analysis were as described by Fahy et al. (15).

T-RFLP analysis of 16S rRNA genes from the upper 1.5 cm of the sediments

DNA extraction from 1 g of sediment used the alternative lysis method of the Ultra Clean Soil DNA isolation kit (MoBio Laboratories Inc., Carlsbad, CA, USA) following manufacturer's instructions, except for an initial vortex of 20 min. Procedures were as described for the tidal biofilms except that PCR amplification of bacterial 16S rRNA genes was carried out with primers TET 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and HEX 1489R (5'-TACCTTGTTACGACTTCA-3') and 35 PCR cycles were used. Fluorescent amplicons were separately digested with restriction enzymes HaeIII, HinfI and HpaII (New England Biolabs). The remaining T-RFLP procedures were as described by Fahy et al. (15), but using Genescan 500 TAMRA internal size standard. T-RFLP profiles were aligned by grouping homologous fragments, and normalized by calculating relative abundances of each terminal restriction fragment (T-RF) from height fluorescence intensity. Normalized T-RFLP profiles were compared by combining data from each restriction enzyme. Statistical analyses were carried out with Primer6 (Plymouth Routines In Multivariate Ecological Research, v 6.1.6) using data from both primers and the three endonucleases. Clustering was based on Bray Curtis similarity, which takes relative abundance into account.

Statistical analysis

The significance of hydrocarbon degradation relative to controls was determined by repeated measures of analysis of variance followed by the post-hoc Tukey's multiple comparison test when $P < 0.05$. The single sample t-test was used to determine whether chrysene/phenanthrene ratios were significantly different. All tests were performed using GraphPad InStat version 3.0a (GraphPadSoftware, San Diego California USA).

Accession numbers

The DNA sequences reported in this study were deposited in the EMBL database under the accession numbers FR852707 to FR852754 for tidal biofilm sequences, FR870040 to FR870075 for DGGE sequences, and FR865969 to FR869630 for pyrosequences.

RESULTS

Analysis of in-situ sediment and water samples

Sediment cores were dominated by silt and clay, and had a water content of $62.6 \pm 0.7\%$. The salinity of overlying water was 38.5‰. The sediments had an obvious biofilm, which largely consisted of diatoms and some euglenoids as seen by fluorescence microscopy (data not shown), and were rich in organic matter ($11.0 \pm 0.4\%$ ash-free dry weight, upper 1.5 cm layer). The most abundant fauna were the polychaete *Nereis diversicolor* and gastropod *Hydrobia* sp. The *in-situ* total petroleum hydrocarbon (TPH) concentration of the top 1.5 cm of the Colne Estuary sediment was $28.4 \pm 1.8 \mu\text{g g}^{-1}$ dry wt. sediment. The levels of hydrocarbons in water samples were much lower ($850 \pm 40 \text{ ng l}^{-1}$). All values are the mean of triplicates $\pm$ standard error. During the experiment the daytime temperature maxima in the water ranged from 23.5 to 33.5°C and night-time minima from 13.5 to 22°C (Fig. S1).

Changes in the hydrocarbon concentration in the mesocosms over 21 days

The most dominant hydrocarbons in the added weathered Forties crude oil were *n*-alkanes ranging from C₁₇ to C₂₅ (58% of aliphatics), and the alkylated forms of naphthalene and phenanthrene (93% of the aromatics) (Table 1). TPH (aliphatic + aromatic hydrocarbons) decreased by 78% in the oiled live sediments (COL) over 21 days (Table 1). Chemical analyses demonstrated significant TPH degradation after 21 days ($F = 32.967$, $p < 0.0001$) while no significant differences were observed between all killed controls ($F = 1.973$; $p = 0.0726$). Although it was impossible to eliminate all microbes by autoclaving sediment and water in killed mesocosms, a minimum of 60% of the TPH loss over 21 days can be attributed to biodegradation (1920 $\mu\text{g g}^{-1}$ TPH loss in the live samples of which 766 $\mu\text{g g}^{-1}$ can be attributed to abiotic loss). Diagnostic ratios (49) provide further evidence that degradation by indigenous microorganisms was the process primarily responsible for the removal of hydrocarbons (Table 1). For example, the $\Sigma\text{chrysene}/\Sigma\text{phenanthrene}$ ratio increased in the live sediment but not in the killed ($t(7) = 3.637$, $p = 0.0083$), reflecting the relative recalcitrance of chrysene and susceptibility of phenanthrene to biodegradation. Aliphatic and aromatic fractions were degraded at roughly equal rates, but those with lower molecular weight were more susceptible to biodegradation and other forms of loss. For example after two days, 31% of the *n*-C₁₂ to *n*-C₁₆ alkane fraction was lost compared with 8% of the *n*-C₂₆ to *n*-C₃₂ fraction ($q = 19.925$, $p < 0.001$; Table 1). Low levels of hydrocarbons were detected in the mesocosm water (Table S1), reflecting the design of the mesocosms which prevented floating oil from entering the tubing.

Effect of crude oil on the bacterial community composition in the 1.5-cm-thick mudflat sediment

Changes in bacterial community composition were analysed by using amplified cDNA from reverse-transcribed 16S rRNA (Figs. S2, S3; Tables S2, S3), and two approaches in which the 16S rRNA genes were amplified directly: T-RFLP (Fig. 4c) and 454 pyrosequencing (Figs. 3, 4a, 4b, S4; Tables 2, S4). Correspondence between sequences identified by RNA-based (DGGE-band sequencing) and DNA-based (pyrosequencing) approaches (see later) attest to the validity of using DNA-based approaches to assess changes in community composition. Such a correspondence has been found in many cases (e.g. 18). The replicate day-12 oiled and non-oiled DGGE profiles are shown in Fig. S3 as an example. Even though triplicate sediment cores were in independent tanks, they had similar profiles (Figs. S2, S3), justifying the pooling of PCR products for subsequent analysis. Rarefaction curves (Fig. S4) demonstrate that the pyrosequencing analysis provided reasonable coverage. PCA plots based on pyrosequencing data (Fig. 4a, b) and cluster analysis of TRFLP data (Fig. 4c) show that the addition of oil had a far bigger impact than time on the bacterial community composition (note the proximity of non-oiled communities CL-2 and CL-21 in Fig. 4a, b, and CL-12 and CL-21 in Fig. 4c). Some change in the oiled community was seen early (day-0 in Fig. 4c), probably because oil had been added for 1.5 days prior to the first measurements on day 0. Divergence in community composition over time was greater in the oiled sediments than those not receiving oil (Fig. 4a, b, c). Nevertheless, overall differences in community composition in the 1.5-cm-thick sediment were modest, as shown by DGGE profiles (Fig. S3) and the high level of similarity in T-RFLP profiles (>80%; Fig. 4c).

Effect of crude oil on tidal biofilm communities

We observed circular biofilms that had detached with the rising tide from their respective sediment core and floated on the water surface. Therefore, DNA was extracted from the tidal biofilms collected on day 22 and T-RFLP analysis of bacterial 16S rRNA genes was

performed. In contrast to the 1.5-cm-thick sediment from the surface of the core, the bacterial communities in the oiled and non-oiled tidal biofilms were extremely different (Fig. 5). Replicate profiles were almost identical (Fig. 5), and so their amplified 16S rRNA genes were pooled, and the main bacteria (and diatoms via their chloroplast 16S rRNA gene sequences) identified by sequence analysis of clone libraries.

Comparison of the main microbial phylotypes detected in the tidal biofilms (day 22) and corresponding 1.5-cm-thick surface sediments (day 21)

The most notable finding was that almost half of the sequences from the oiled tidal biofilms came from an *Alcanivorax* sp., which was not detected in the non-oiled biofilm (Table 3). *Alcanivorax* was detected in the corresponding oiled sediment at day 21 (Table 2), but at 72-times lower relative sequence abundance. Phylogenetic analysis showed that *Alcanivorax* populations from the tidal biofilms and the 1.5-cm-thick sediments were distinct (Fig. S5; clusters A1 and A2, respectively). Those from the tidal biofilms were mostly identical in partial 16S rRNA gene sequence to the type species *Alcanivorax borkumensis*, whereas those in the sediment had only 93% identity to *A. borkumensis* and 96-98% identity to several partially characterized *Alcanivorax* strains.

In addition, 11% of clones from the day-22 oiled tidal biofilm had 99% identity to *Oceanospirillales* strain ME113, previously enriched with hexadecane from a Sicilian harbour seawater-sediment sample (16) (Table 3; Fig. S6). As with *Alcanivorax*, this phylotype was undetected in the non-oiled tidal biofilms (Table 3), but an identical phylotype constituted 1% of the sequences from the 1.5-cm-thick oiled sediment community at day 21 (Table 2; Fig. S6). Other phylotypes enriched in the day-21 oiled 1.5-cm-thick sediment, e.g. *Oleibacter*, *Marinobacter* and *Cycloclasticus*, were not detected in the oiled tidal biofilms (Tables 2, 3).

Comparison of the phylotypes differentially detected in the oiled and corresponding non-oiled 1.5-cm-thick surface sediments

Although differences in DGGE profiles from the 1.5-cm-thick sediments were modest, a few bands from oiled and non-oiled sediments were differentially abundant. These bands from day-12 DGGE profiles were sequenced (Fig. S3; Tables S2, S3). Two bands from *Oceanospirillales* were detected only from the oiled sediments: band COLa had 99% identity to strain ME113 previously observed in the oiled floating biofilm, and COLb1 had 96% identity to the alkane-degrader *Oleibacter marinus* (45). Various cyanobacteria from the order Oscillatoriales were detected in bands that were more intense in the oiled sediment (Fig. S3; Table S2). However, many bands had sequences from numerous phylogenetic groups, a common occurrence in such diverse sediments, that potentially masks changes in bacterial community composition (Table S3). Therefore, pyrosequencing was carried out on pooled replicate PCR products from five samples with a view to gain finer resolution and clearer insights into the influence of oil on the sediment microbial community (Tables 2, S4). A total of 3731 sequences longer than 133 bp were analysed.

Figure 3 summarizes the changes in relative proportion of higher taxonomic groups, and most notably shows the enrichment of cyanobacteria, and to a lesser extent diatoms, at day 21 in the oiled sediments, corresponding with fluorescence microscopic observations (data not shown). In order to explore changes at the genus level, sequences were sorted into clusters with $\geq 95\%$ identity, and BLAST analysis was performed on a representative from each cluster that contained more than five sequences (representing 44% of all sequences). Table S4 shows a high level of similarity between oiled and non-oiled sediment samples, supporting the DGGE analysis (Fig. S3).

Nevertheless, some phylotypes were more frequently detected in the oiled sediments (Table 2). In particular, and corroborating the day-12 DGGE analysis (Fig. S3; Table S3), there was an increase in abundance of cyanobacteria from the orders Oscillatoriales and Stigonematales, especially at day 21 (Table 2). Chloroplast 16S rRNA genes from a diatom of the order Bacillariales were more abundant in oiled sediments at day 21, but not at day 2. Five clusters that were only or predominantly detected in oiled sediments consisted of known hydrocarbonoclastic Gammaproteobacteria: 98% identity to several strains of *Alcanivorax* (but only 93% identity to the type species *A. borkumensis*); 98% identity to *Marinobacter* spp.; 95% identity to *Oleibacter marinus*; 99% identity to Oceanospirillales strain ME113; and 100% identity to the type species of *Cycloclasticus* (Table 2). At day 21, these sequences from hydrocarbonoclastic bacteria made up 2% of sequences from the oiled sediment whereas they were undetected in the non-oiled sediment.

DISCUSSION

The sediments used in this study have medium-level TPH contamination (37). However, detailed analysis of the hydrocarbon fingerprint showed a dominance of C₂₅, C₂₇, C₂₉ and C₃₁ *n*-alkanes (determined as biowaxes), which suggested a greater contribution of hydrocarbons from detritic organic matter and vascular plants than petrogenic sources and consequently oil contamination (36). This observation was further supported by the absence of the C₃₀-hopanes, which are biomarkers for petrogenic hydrocarbons (49).

Factors influencing hydrocarbon degradation

Although many factors influence rates of hydrocarbon degradation, our results are in agreement with those from chronically polluted muddy (35) and sandy (33) sediments.

Conversely, data from other investigations (8, 19) suggest that the extent of hydrocarbon depletion that we observed by day 8 was high. The unusually high temperatures for a UK summer (Fig. S1) would have enhanced biodegradation. The pattern of hydrocarbon degradation, with loss of lower molecular weight hydrocarbons was typical (22). Also, TPH loss was faster at the beginning of the experiment (Table 1), indicative of an acclimated microbial community, and several factors probably contributed to the deceleration of degradation after day 8, including loss of more biodegradable compounds and sorption of the more recalcitrant hydrocarbons. Availability of fixed nitrogen and phosphorous often limit oil biodegradation (38), and may have done so in this study, as evidenced by a rapid decrease in inorganic nitrogen concentrations after day 2 in the oiled mesocosms compared with those that received no oil (data not shown).

Reasons for modest oil-induced changes in bacterial and diatom community composition in the 1.5-cm-thick mudflat sediment

The upper 1.5 cm of the sediment housed a very diverse and heterogeneous bacterial community. Very few phylotypes were less abundant in oiled sediments (Table S4), which may in part be because deeper microbes had limited contact with oil that was retained by the surface biofilm (8). Although holes created by *Nereis diversicolor* were seen, oil ingress may have been minimal: for example Grossi et al. found that oil was undetected below 0.3 cm at 2 months in a bioturbated muddy sub-tidal sediment (19). Even if oil had penetrated further, sorption of hydrocarbons to organo-clays would reduce bioavailability and thus toxicity. However, a general resistance of the microbial community to oil is assumed, because proportions and types of surface-dwelling diatom taxa that came into direct contact with oil were very similar to those from non-oiled sediments. The presence of oil-tolerant microbes,

as well as the detection of hydrocarbonoclastic *Alcanivorax* spp. in the non-oiled sediment at day-0 (Table 2), can be explained by prior exposure to hydrocarbons in the *in-situ* sediment.

The modest oil-induced community change observed here contrasts starkly with that seen by dos Santos et al. where Chromatiales decreased in relative abundance approximately ten-fold over an equivalent period (11). This can be attributed to their mixing of the mangrove sediment with oil thus destroying the chemocline, and the intimate contact with oil that would make microbes more susceptible to both its direct toxic effects and its indirect effects, such as reducing available oxygen owing to competition with nearby oil degraders.

Oil-influenced taxa

The major effect of oil addition on microbial community composition was an increase in cyanobacteria, especially apparent at day 21 (Fig. 3). The possibility that cyanobacteria degrade petroleum hydrocarbons is unlikely (see 1). Other explanations are that a cyanobacteria-specific grazer has been inhibited by oil, or diatoms that may be more susceptible to the toxic effects of oil, leave an unoccupied niche. Alternatively, the depletion of ammonia and nitrate may have selected for nitrogen-fixing cyanobacteria (currently under investigation).

Many sequences were detected that belong to microbes with the potential to degrade hydrocarbons. For example, although *Marinobacter* is recognized as a common oil-degrading bacterium and was enriched in oiled 1.5-cm-thick sediments, it was also prevalent in the non-oiled tidal biofilms, reflecting the broad nutritional versatility within the genus (12). Here, we restrict the discussion to phylotypes for which there is strong evidence for hydrocarbon degradation, and that were greatly enriched in oiled mesocosms (especially from the tidal biofilms).

Sequences that were 100% similar to diverse *Cycloclasticus* species, including the type species, were found exclusively in the three oiled sediments examined by pyrosequencing. Since *Cycloclasticus* spp. are PAH-degrading specialists (14) it can be inferred that this is their role in the sediments here, and supports their widespread oil/PAH-induced marine distribution, e.g. in marine and estuarine waters and occasionally in coastal sediments (6, 11, 22, 28, 29, 32, 43, 51, 52).

Several sequences that were 95 to 96% similar to those from *Oleibacter marinus*, which specifically degrades *n*-alkanes (44, 45), were found by DGGE and pyrosequencing in oiled 1.5-cm-thick sediments only, particularly at day 12. Within this phylogenetic cluster (O-1 in Fig. S6), two phylotypes were more closely related to *Oleibacter* and one was identical to clones previously detected in enriched crude-oil-amended water from the Thames Estuary (6), thus supporting the potential role in hydrocarbon biodegradation of these novel phylotypes belonging to the Oceanospirillales.

Comparison of microbial communities in the sediment and on the tidal biofilms

Neither *Cycloclasticus* nor *Oleibacter*-related sequences were detected in the tidal biofilms. In contrast, *Alcanivorax* and phylotypes related to *Oceanospirillales* strain ME113 were both abundant in the oiled tidal biofilms and present in oiled 1.5-cm-thick sediments. Strain ME113 was isolated from a sediment-seawater mix from a Mediterranean harbour with *n*-hexadecane as sole source of carbon (16). Cluster O-2 (Fig. S6), containing sediment and tidal biofilm sequences as well as strain ME113, also includes a phylotype enriched in diesel-supplemented Mediterranean seawater mesocosms (25) and a phylotype that dominated Arctic seawater in which *Alcanivorax* was also abundant (yet there was no mention of hydrocarbon pollution at this location (4)). Therefore, we can reasonably assume that these Oceanospirillales bacteria degrade some component of crude oil. Despite having obtained

over 100 diverse hydrocarbon-degrading isolates from a similar environment (Sanni and McGenity, unpublished data), none was related to these ME113-like clones. Indeed strain ME113 has proven difficult to maintain in culture (Yakimov pers. comm.). Given their widespread association with, or enrichment in, oil-polluted environments, it will be important to culture and investigate ME113-like Oceanospirillales, as well as many other uncultivated representatives of this order which probably have a role in oil degradation and have been found in the Thames Estuary (6, 28), Mediterranean Sea (25, 51, 52), and around the oil escaping from the Deepwater Horizon rig in the Gulf of Mexico (21).

The dominant phylotype in the oiled tidal biofilms, represented by almost half of the sequences, had ~100% 16S rRNA sequence identity to *Alcanivorax borkumensis* SK2. This phylotype (A-1 in Fig. S5) was absent from the non-oiled biofilm, and although *Alcanivorax* was detected in oiled 1.5-cm-thick sediments, it represented a distinct phylotype (A-2 in Fig. S5). Thus, in contrast to the ME113-like Oceanospirillales, distinct biofilm and sediment *Alcanivorax* ecotypes were found. The biofilm phylotype is represented by an organism about which we have substantial understanding based on post-genomic studies (40, 52). McKew et al. showed that *A. borkumensis* was the dominant microbe in microcosms containing branched alkanes from the nearby Thames Estuary (28), but it is capable of degrading a wide range of *n*-alkanes, and is frequently the most abundant microbe found associated with marine oil spills (6, 22, 24, 29, 39, 44, 51, 52). It is surprising that this phylotype was found only in the tidal biofilms, and was not in the upper 1.5 cm of sediment. The *Alcanivorax* sequences found in the 1.5 cm sediment were most closely related (98 and 97% 16S rRNA identity, respectively) to strain GPM2532 isolated from a mangrove sediment (5) and PA2 from 0-3 cm deep intertidal sediment (34), indicative of a phylotype preferentially adapted to living within muddy sediments. Nutrient amendments have previously been suggested to select for different phylotypes of *Alcanivorax* (22). Elucidating the nature of such niche

partitioning requires more detailed experiments, and understanding habitat preference will be important in cases where cultures of *Alcanivorax* spp. are considered for bioaugmentation (29).

The obvious advantage to aerobic hydrocarbon-degrading bacteria living in a biofilm is that it allows constant contact, in an oxygen-rich environment, to the oil that detaches at high tide from the sediment and floats on the water's surface. Close proximity to diatoms in the biofilm will also ensure a supply of photosynthetically derived oxygen which is a key reactant and terminal electron acceptor for alkane degradation by *Alcanivorax borkumensis*. The fact that *Alcanivorax* sp. constitutes 49% of the tidal biofilm community suggests that it plays a role in biofilm formation. However, biofilms also formed in the absence of oil and *Alcanivorax*, and so the contribution by species common to both oiled and non-oiled biofilms must be significant, notably diatoms that produce copious amounts of EPS (20) – the additional possibility that diatom EPS emulsifies hydrocarbons deserves investigation. *Alcanivorax* spp. have been shown to inhabit the phycosphere of algae such as the dinoflagellate *Gymnodinium catenatum* (17), and even to reduce the lag phase and enhance the maximum chlorophyll fluorescence of the cyanobacterium *Prochlorococcus* by means of diffusible molecules (42). Here, *Alcanivorax* will most likely benefit the diatoms by consuming toxic hydrocarbons, and when there is no pollution, might consume the natural hydrocarbons, such as isoprene, produced by diatoms (2).

Multiple adaptations to tolerate ultraviolet light (40) may have also contributed to the dominance of *A. borkumensis* phylotypes in the tidal biofilms. However, it is possible that there was a succession of hydrocarbon-degrading bacteria on the tidal biofilms in addition to *Alcanivorax* and Oceanospirillales strain ME-113 types, particularly given the known ability of *Marinobacter hydrocarbonoclasticus* to form biofilms (46) and of diverse hydrocarbon degraders to inhabit the surface of microalgae (1).

Detachment of surface biofilms is common in intertidal sediments, with ~15% of benthic chlorophyll *a* (indicative of diatom biomass) being washed away within 30 minutes of tidal cover in the Colne Estuary (20). Thus, the ability of aerobic hydrocarbonoclastic bacteria to live in a surface biofilm potentially provides another selective advantage by allowing dispersal on the tide, resulting in the tidal biofilms being stranded on the tide line, which is precisely where newly deposited or redistributed oil, and thus a fresh source of carbon and energy, would also appear.

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FIGURE LEGENDS

Figure 1: Location map of sampling site on the Colne Estuary (Essex, UK; 51°50.5'N, 0°58.4'E) used for the oil-spill mesocosms.

Figure 2: Schematic representation of the experimental tidal mesocosms. (a) Nine polypropylene containers (32.5 × 42 × 25.5 cm) holding 20 sediment cores (9.8 cm high, 6.5 cm diameter) were placed above reservoir containers. Three different treatments were carried out in triplicate as follows: (i) autoclaved sediment cores with Forties crude oil (Colne Oil Killed); (ii) sediment cores with Forties crude oil (Colne Oil Live); and (iii) sediment core without oil (Colne Live). (Air temperature probe: (T_{air}); temperature probe in: sediment (T_{sub1}), water reservoir tank (T_{sub2}) and water tank containing cores (T_{sub3}); tube solar energy sensors (Solm); spot sensors for solar energy in the photosynthetically active waveband (QS). (b) Side view of the upper container holding the sediment cores and the lower reservoir, illustrating the flow of seawater. At low tide the pump was off and seawater drained to the level of the tube connected to the pump. At high tide the pump was on and seawater flowed continuously through a tube passing through the hole. The tube was curved downwards into the upper tank to prevent transfer of oil from the upper tank to the reservoir.

Figure 3: Proportion of the higher taxa in mudflat mesocosms (top 1.5 cm of sediment), based on 454 pyrosequencing analysis of bacterial 16S rRNA genes (Greengenes analysis; RDP classification). Left: bacterial communities in non-oiled sediments. Right: bacterial community in oiled sediments. CL-2: non-oiled sediment, day 2 (899 sequences); COL-2: oiled sediment, day 2 (258 sequences); COL-12: oiled sediment, day 12 (321 sequences); CL-21: non-oiled sediment, day 21 (761 sequences); COL-21: oiled sediment, day 21 (1492 sequences). Actino = Actinobacteria; Bactero = Bacteroidetes; Chloro = Chloroflexi; Diatom

= Bacilliophycaceae; AlphaP = Alphaproteobacteria; GammaP = Gammaproteobacteria;
DeltaP = Deltaproteobacteria.

Figure 4: Principal Component Analysis of 454 pyrosequencing data and cluster analysis of TRFLP data from sediment samples.

Data are based on directly amplified 16S rRNA genes from Bacteria. (a) PCA plot based on P1 and P2 components. (b) PCA plot based on P1 and P3 components. The first principal component (P1) explains 35.8% of the variation between the environments, the second (P2) 26% and the third (P3) 23%. PCA was conducted using the UniFrac metric algorithm and based on a Neighbour-Joining phylogenetic tree. (c) Cluster analysis based on Bray Curtis similarity taking into account square root transformation of T-RF relative abundances (based on the mean height of the T-RFs). Oiled sediments are indicated by closed squares and non-oiled sediments by open squares.

Figure 5. T-RFLP profiles of triplicate tidal biofilms harvested in oiled (biofilm-COL) and non-oiled (biofilm-CL) mesocosms on the last day of the experiment (day 22).

The profiles were obtained with primer 63F and restriction enzyme AluI; for clarity, data from primer 1389R are not shown. The peak height represents the relative abundance of each T-RF, and the horizontal scale represents the T-RF length in nucleotides. The highlighted T-RF peak (size 35nt) from the oiled tidal biofilms dominates each of the three communities. In the non-oiled tidal biofilms however, a double peak is observed at this position. By correlating in-silico T-RF positions from cloned sequences, it is clear that the 35nt T-RF in the oiled floating biofilm comes from *Alcanivorax*, while the double peak at a similar position in the non-oiled biofilm comes from various other groups, including Gammaproteobacteria, Bacteroidetes, as well as species distantly related to cultured organisms.

TABLES

Table 1 Biodegradation and weathering indices change in the core sediments (upper 1.5 cm layer)

Hydrocarbons / ratio indices	Concentration (µg/g d.w. sediment)								Percentage loss (%) and ratio changes relative to T0						
	COL sediment							COK ^a	COL sediment						COK ^a
	T0	2 days	5 days	8 days	12 days	16 days	21 days	21 days	2 days	5 days	8 days	12 days	16 days	21 days	21 days
C ₁₂ -C ₁₆ ^b	372	255	210	134	91	64	41	189	31	43	64	75	83	89	49
C ₁₇ -C ₂₅ ^b	1005	857	707	428	405	322	208	708	15	30	57	60	68	79	30
C ₂₆ -C ₃₂ ^b	253	232	193	139	122	92	57	165	8	24	45	52	64	78	35
C ₃₃ -C ₃₇ ^b	87	84	79	72	63	61	32	84	3	10	17	28	30	63	3
Total aliphatic	1717	1429	1188	773	681	538	338	1145	17	31	55	60	69	80	33
C17/Pristane	2.32	2.12	2.09	1.91	1.49	1.22	1.15	1.85	0.9	0.8	0.6	0.5	0.5	0.5	0.8
C18/Phytane	2.45	2.32	2.29	2.12	1.82	1.63	1.34	2.14	0.9	0.9	0.7	0.7	0.5	0.5	0.9
Naphthalene	0.30	0.25	0.24	0.19	0.15	0.12	0.04	0.16	16	20	37	50	60	87	48
C1-napht ^c	67.7	48.1	39.7	20.3	15.3	12.3	11.2	37.9	29	41	70	77	82	83	44
C2-napht ^c	199.3	148.8	111.3	97.4	78.2	56.9	42.9	111.6	25	44	51	61	71	78	44
C3-napht ^c	253.8	222.8	165.3	123.9	109.4	78.4	69.6	203.6	12	35	51	57	69	73	20
Acenaphthylene	3.20	2.50	1.40	1.21	0.97	0.74	0.62	1.61	22	56	62	70	77	81	50
Acenaphthene	0.35	0.33	0.22	0.18	0.08	0.07	0.05	0.19	4	37	49	77	80	86	46
Fluorene	1.13	0.73	0.63	0.43	0.34	0.22	0.19	1.00	35	44	62	70	81	83	11
C1-fluorene	1.29	0.92	0.80	0.71	0.62	0.53	0.41	1.13	29	38	45	52	59	68	13
C2-fluorene	1.96	1.14	0.87	0.79	0.57	0.47	0.41	1.64	42	56	60	71	76	79	17
Dibenzothiophene	0.52	0.37	0.30	0.27	0.22	0.20	0.18	0.48	29	42	48	58	62	65	7
C1-Dibenzo	1.28	0.91	0.87	0.78	0.65	0.45	0.42	1.21	29	32	39	49	65	67	6
C2-Dibenzo	0.55	0.42	0.36	0.32	0.29	0.26	0.24	0.49	24	35	42	47	53	56	10
Phenanthrene	2.33	1.88	1.19	0.96	0.65	0.49	0.42	2.04	20	49	59	72	79	82	13
C1-phen/anth ^c	20.40	16.81	12.55	10.86	9.59	8.11	6.10	18.02	18	38	47	53	60	70	12
C2-phen/anth ^c	70.35	63.80	58.26	47.07	35.98	28.17	24.90	60.11	9	17	33	49	60	65	15
C3-phen/anth ^c	17.80	14.23	12.46	11.15	10.57	9.40	8.34	15.35	20	30	37	41	47	53	14
Fluoranthene	18.60	13.60	11.09	10.60	8.11	7.30	6.98	16.86	27	40	43	56	61	62	9
Pyrene	1.60	1.43	1.22	1.09	0.98	0.86	0.84	1.42	11	24	32	39	46	48	11
C1-pyr ^c	2.00	1.87	1.81	1.75	1.60	1.45	1.32	1.80	6	10	13	20	28	34	10
C2-pyr ^c	2.62	2.41	2.33	2.29	2.20	2.16	2.13	2.42	8	11	13	16	18	19	8
Chrysene	2.61	2.47	2.41	2.32	2.28	2.22	2.04	2.52	5	8	11	13	15	22	3
C1-Chrysene ^c	0.62	0.59	0.55	0.53	0.51	0.49	0.49	0.60	5	11	15	18	21	21	3
C2-Chrysene ^c	0.48	0.47	0.46	0.45	0.42	0.40	0.38	0.47	2	4	6	13	17	21	3

Total aromatic	671	547	426	336	280	212	180	483	18	36	50	58	68	73	28
Σ Chrysene/ Σ Phen	0.03	0.04	0.04	0.05	0.06	0.07	0.07	0.03	1.1	1.2	1.4	1.7	2.0	2.2	0.9
Σ Diben/ Σ Phen	0.02	0.02	0.02	0.02	0.03	0.02	0.03	0.02	0.8	0.8	0.9	1.0	1.0	1.1	1.1
TPH^d	2388	1976	1615	1109	961	750	518	1628	17	32	54	60	69	78	32

Each value represents the mean amount of hydrocarbon groups or compounds from triplicate samples. ^a Killed controls used to estimate abiotic loss. ^b

Alkanes are abbreviated by the number of carbon atoms in chain. ^c C1 to C3 represent carbon number of alkyl groups in alkylated PAH homologues. ^d

Total petroleum hydrocarbons (TPH) equal to the sum of aliphatic and aromatic compounds.

Table 2 Phylogenetic clusters enriched in the upper 1.5 cm of mudflat sediment after oil addition, based on 454 pyrosequencing analysis

Cluster	Higher taxon gp	Order/ Family	Close relative (accession number)	% id	CL 2	COL 2	COL 12	CL 21	COL 21
					Percentage sequences from each sample				
39	Diatom	Bacillariales	Diverse (AY221721)	99	0.33	0.00	0.31	0.00	0.80
22	Cyano	Oscillatoriales	<i>Lyngbya</i> (AY768394)	98	0.00	0.39	0.00	0.39	4.09
34	Cyano	Oscillatoriales	Diverse (AM398790)	97	0.00	0.00	0.00	0.00	0.80
61	Cyano	Oscillatoriales	Diverse (AM398790)	98	0.00	0.00	0.00	0.00	0.40
556	Cyano	Oscillatoriales	<i>Spirulina</i> (AJ635436)	100	0.00	0.00	0.62	0.00	0.60
31	Cyano	Stigonematales	<i>Mastigocladopsis</i> (AJ544079)	98	0.00	0.00	0.00	0.00	3.02
167	GammaP	Alcanivoracaceae	<i>Alcanivorax</i> (AJ871931)	98	0.11	1.55	2.18	0.00	0.67
524	GammaP	Alteromonadales	<i>Marinobacter</i> (GU361128)	98	0.00	0.00	1.25	0.00	0.13
321	GammaP	Chromatiales	Diverse (FN293071)	90	0.00	0.39	0.00	0.00	0.54
469	GammaP	Oceanospirillales	<i>Oleibacter</i> (AB435651)	95	0.00	0.00	1.87	0.00	0.47
477	GammaP	Oceanospirillales	strain ME113 (AJ302700.1)	99	0.00	1.55	2.18	0.00	0.87
519	GammaP	Thiotrichales	<i>Cycloclasticus</i> (L34955)	100	0.00	1.16	1.56	0.00	0.20
109	AlphaP		strain SOGA19 (AJ244796)	98	0.00	0.00	0.00	0.00	0.47

This table contains a subset of Table S4, showing those clusters enriched in the oiled mesocosms (COL, shaded columns). Sequences (3731) were analysed from the five samples by using CD-HIT to generate clusters with $\geq 95\%$ identity. A representative sequence was analysed by BLAST for those clusters containing ≥ 6 sequences (43.6% of sequences). Cluster numbers are assigned by CD-HIT. Higher taxonomic groups (higher taxon gp) are: Diatom (Bacilliophycaceae), Cyano (Cyanobacteria), GammaP (Gammaproteobacteria), AlphaP (Alphaproteobacteria). Closest relatives are only given when informative. ‘% id’ represents the percentage identity in 16S rRNA gene over 133 nucleotides.

Table 3 Comparison of the main taxa detected in 16S rRNA gene clone libraries from the oiled and non-oiled tidal biofilms at day-22

Higher taxon gp	Closest relative (accession number)	% id	biofilm-COL	biofilm-CL
Percentage sequences from each sample				
Diatom	<i>Nitzschia</i> PLAT 907 (EU346643)	96-98	13.6	10.2
	LIQUIDNW12H04 (EU346457)	97.4	0.0	5.1
GammaP	<i>Alcanivorax borkumensis</i> (AM286690)	100	48.5	0.0
	<i>Oceanospirillales</i> ME113 (AJ302700.1)	97.3	10.6	0.0
	<i>Marinobacter</i> EN6 (EF660754)	99.6	0.0	5.1
AlphaP	<i>Rickettsia typhi</i> Wilmin (AE017197)	91.6	0.0	5.1
DeltaP	<i>Pelobacter</i> A3b3 (AJ271656)	98.0	0.0	10.2
	<i>Bacteriovorax</i> NF2 (EF092442)	89.7	0.0	5.1
Bactero	<i>Flexibacteraceae</i> M 1368 (AM990675)	99.5	0.0	5.1

From the oiled tidal biofilms (biofilm-COL, shaded column) 66 clones were analysed, 5 doubles and 8 singletons are not shown; while from the non-oiled tidal biofilms (biofilm-CL) 59 clones were analysed, 4 doubles and 24 singletons are not shown. Representative clones were sequenced from each RFLP group. Higher taxonomic groups (higher taxon gp) are: Diatom (Bacilliophycaceae), GammaP (Gammaproteobacteria), AlphaP (Alphaproteobacteria), DeltaP (Deltaproteobacteria), Bactero (Bacteroidetes). ‘% id’ represents the percentage identity in 16S rRNA gene over 425 to 492 nucleotides.



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Central role of dynamic tidal biofilms dominated by aerobic hydrocarbonoclastic bacteria and diatoms in the biodegradation of hydrocarbons in coastal mudflats

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