Hydrocarbon-degradation and MOS-formation capabilities of the dominant bacteria enriched in sea surface oil slicks during the *Deepwater Horizon* oil spill

Tony Gutierrez,[1],* Gordon Morris,[2] Dave Ellis,[3] Bernard Bowler,[4] Martin Jones,[4] Karina Salek,[1] Barbara Mulloy,[5] Andreas Teske,[6]

[1] Institute of Mechanical, Process and Energy Engineering (IMPEE), School of Engineering and Physical Sciences, Heriot-Watt University, Edinburgh, UK; [2] Department of Chemical Sciences, School of Applied Sciences, University of Huddersfield, Huddersfield, UK; [3] Institute of Chemical Sciences (ICS), School of Engineering and Physical Sciences, Heriot-Watt University, Edinburgh, UK; [4] School of Civil Engineering and Geosciences, Newcastle University, Newcastle upon Tyne, UK; [5] Laboratory for Molecular Structure, National Institute for Biological Standards and Control (NIBSC), Hertfordshire, UK; [6] Department of Marine Sciences, University of North Carolina, Chapel Hill, North Carolina, USA.

*Correspondence to: Dr. Tony Gutierrez

Institute of Mechanical, Process & Energy Engineering (IMPEE), School of Engineering & Physical Sciences, Heriot-Watt University, Edinburgh EH14 4AS, U.K.

Email: tony.gutierrez@hw.ac.uk

Tel: +44 (0)131 451-3315

Running title: Dominant bacteria enriched at Deepwater Horizon

Keywords: hydrocarbon-degrading bacteria; Deepwater Horizon; Gulf of Mexico; exopolysaccharides (EPS); crude oil; biodegradation

Abstract

A distinctive feature of the Deepwater Horizon (DwH) oil spill was the formation of significant quantities of marine oil snow (MOS), for which the mechanism(s) underlying its formation remain unresolved. Here, we show that *Alteromonas* strain TK-46(2), *Pseudoalteromonas* strain TK-105 and *Cycloclasticus* TK-8 – organisms that became strongly enriched in sea surface oil slicks during the spill – contributed to the formation of MOS and/or dispersion of the Macondo oil. In roller-bottle incubations, *Alteromonas* cells and their produced EPS yielded MOS, whereas *Pseudoalteromonas* and *Cycloclasticus* did not. Interestingly, the *Cycloclasticus* strain was able to degrade *n*-alkanes concomitantly with aromatics within the complex oil mixture, which is atypical for members of this genus. Our findings, for the first time, provide direct evidence on the hydrocarbon-degrading capabilities for these bacteria enriched during the DwH spill, and that bacterial cells of certain species and their produced EPS played a direct role in MOS formation.

Introduction

The Deepwater Horizon (DwH) blowout of April 20, 2010 is recorded as the worst oil spill in US history. Estimates of the overall magnitude of the release vary, with recent figures reporting approximately 3.19 million barrels (134 million gallons) of oil (U.S. v. BP et al., 2015), and at least 250,000 metric tonnes of natural gas, largely methane, released into the Gulf of Mexico over a period of 87 days (Valentine et al. 2010; Joye et al. 2011). Based on its magnitude, difficulty and complexity of the clean-up response, the spill was marked as one of the worst in the history of the oil and gas industry (Lubchenco et al. 2012). Two distinctive features set the DwH spill apart from other oil spills at sea. The first was the formation of a hydrocarbon-enriched plume (Du & Kessler, 2012; Ryerson et al. 2012) that became entrained as a lens at a depth of 1000-1300 m depth within the water column (Camilli et al., 2010; Diercks et al., 2010). Whilst this deepwater plume had, from the outset of the spill, attracted intense interest from the scientific community in tracking its movement, analysing its physicochemical properties and evolving microbial community, the formation of unprecedented quantities of marine oil snow (MOS) – the other distinctive feature of the DwH spill – gradually gained the interest of the scientific community, with the first reports to emerge on MOS by 2012 (Passow et al. 2012; Ziervogel et al. 2012). MOS is defined as mucilaginous floating organic matter with a "fluffy" off-white appearance, and which distinctively contains associated oil droplets. MOS formation and its impact to the Gulf, and during other spills where it was observed to have formed (i.e. *Ixtoc-I* and *Tsesis* oil spills), has received considerable attention, with more than 50 of studies that consider MOS formation following the DwH spill (Vonk et al, 2015; Daley et al, 2016; Passow, 2016).

The large quantities of MOS observed during the DwH spill were observed during the first research cruise on *R/V Pelican* to the spill site in early May, 2010, and were frequently encountered around the vicinity of surface oil slicks (Niu et al. 2011; Passow et al. 2012) and within deep water oil plumes (Niu et al. 2011). By June 2010, a little over a month after the onset of the spill, MOS was no longer visible on surface waters in the Gulf of Mexico, as it had subsequently sedimented to

the ocean floor (Hollander et al. 2012; Joye et al. 2014). MOS sedimentation has also been

suggested to have originated from the deepwater plume (Valentine et al., 2014). Significant

hydrocarbon deposition to the seafloor was observed within 20 km of the spill site (Brooks et al.,

2015; Romero et al., 2015; Romero et al., 2017; Stout et al., 2017) and including to the northern

Gulf of Mexico (White et al., 2012; Montagna et al., 2013; Valentine et al., 2014; Chanton et al.,

2015) as a product of MOS formation (Brooks et al. 2015). Estimates for the amount of weathered

oil residues that were transported to the seafloor is comparable between studies, from 1.8-14%

(Valentine et al. 2014) to 0.5-9% (Chanton et al. 2015), though still uncertain. Now, just over seven

years on, the full environmental impact of this MOS-mediated oil sedimentation remains

unresolved.

Although conjecture still surrounds what triggered MOS formation during the DwH spill,

the prevailing evidence indicates that it was directly associated with the massive influx of crude oil

into the Gulf of Mexico. In roller-bottle experiments performed under conditions simulating the

Gulf spill by adding weathered oil collected from the sea surface near the Macondo wellhead,

Ziervogel et al. (2012) elegantly demonstrated the importance of the oil in MOS formation, and that

these amorphous aggregations could act as hotspots of microbial oil-degrading activity that

significantly influenced carbon flux in surface oil slicks at DwH. Passow et al. (2012) provided

further insight on the complexity of biological interactions that contributed to the formation of

MOS, and Bælum et al. (2012) described the formation of flocs (synonymous with MOS) in

incubations with seawater and oil from DwH, and showed *Colwellia* was a dominant member of the

flocs formed. These studies in the Gulf of Mexico and elsewhere have explored the genesis of MOS

formation, revealing the involvement of bacteria, oil, dispersants, mucilaginous polymers (e.g. TEP,

EPS) and possibly also eukaryotic phytoplankton (Gutierrez et al. 2013a; Arnosti et al. 2016; Duran

Suja et al. 2017; Passow et al., 2012; Fu et al. 2014; Passow, 2016). Whether any one of these

biological contributors plays a protagonist role in triggering MOS formation remains

unsubstantiated. During the DwH spill, their ubiquity in the Gulf water column suggests they had at least contributed in concert in the formation of MOS.

Marine snow particles are held together by carbohydrate-based polymers, such as transparent extracellular particles (TEP) and/or extracellular polymeric substances (EPS), that can be produced in large quantities by phytoplankton and bacteria. Certain groups of bacteria in the ocean are recognized for producing significant quantities of EPS that contribute to the total dissolved organic matter (DOM) pool in the ocean (Azam, 1998). A large fraction of this bacterial-derived EPS consists of glycoprotein (Long and Azam, 1996; Verdugo et al. 2004), which coincidentally reflects the chemical composition found in MOS that formed in surface and plume waters at DwH (Bælum et al. 2012; Passow et al. 2012; Ziervogel et al. 2012). It can be hypothesized that indigenous groups of EPS-producing bacteria that became enriched during the DwH spill had contributed, via an as yet unknown mechanism(s), to the formation of significant quantities of MOS observed during the spill. Since no methods currently exist that can match any type of EPS in an environmental sample to its biological source, one method to ascertain whether EPS produced by a bacterial group enriched during the DwH spill may had contributed to the formation of MOS is to study them in pure culture. Using roller-bottle incubations under conditions simulating oil-contaminated sea surface water, we previously showed that EPS produced by *Halomonas* sp. TGOS-10 – a hydrocarbon-degrading bacterium that had become enriched in sea surface oil slicks during the DwH spill – could trigger the formation of MOS in the presence of crude oil (Gutierrez et al. 2013a). Other studies also described MOS formation in similar laboratory-based incubations with seawater (Bælum et al. 2012; Passow et al. 2012; Ziervogel et al. 2012), but the role of EPS in this respect was not investigated. In this study, we test the hypothesis that other hydrocarbon-degrading bacteria that became enriched in surface oil slicks during the spill were involved in inducing the formation of MOS, as well as participating in the emulsification and degradation of the Macondo oil. MOS formation was evaluated in oil-amended roller bottle incubations in the laboratory over a period of 14 days using constant gentle turbulence to simulate

conditions near the sea surface. We also determined the range of hydrocarbons in Macondo oil that these strains are capable of degrading, and we isolated the EPS produced by one of the strains in order to analyse its chemical characteristics and infer on its role in MOS formation and emulsification of the oil.

Materials and Methods

Microorganisms used in this study

Alteromonas sp. strain TK-46(2) was originally isolated from a sea surface oil slick sample collected during a research cruise on RV *Pelican* on May 5th of 2010, ca. 0.86 miles from the site of the DwH blowout (28° 44.175' N, 88° 22.335' W). *Pseudoalteromonas* sp. strain TK-105 had been isolated from a deepwater plume sample collected at 1170 m depth (28° 41.686' N, 88° 26.081' W) during a subsequent cruise on the RV *Walton Smith* on 31st May 2010. The strains were selected for use in this study based on their ability to produce EPS, to degrade hydrocarbons, and because they were identified as enriched taxa in oil-contaminated water column samples collected during the DwH oil spill (Gutierrez et al. 2013a,b). *Cycloclasticus* sp. strain TK-8 was isolated from the sea surface oil slick and included in this study because, whilst it too was found heavily enriched in sea surface oil slicks and the deepwater plume (Gutierrez et al. 2013b), this strain does not produce EPS and therefore served as a useful reference organism to compare with the EPS-producing strains TK-46(2) and TK-105. In addition, it had not previously been determined, by empirical investigation, the range of hydrocarbons in the Macondo crude oil that each of these strains is able to degrade, so was assessed here.

Hydrocarbon analysis

To determine the hydrocarbon species in Macondo crude oil that strains TK-8, TK-46(2) and TK-105 can degrade, a synthetic seawater medium, ONR7a (Dyksterhouse et al. 1995) was used and amended with surrogate Macondo crude oil (from the Marlin platform) as the sole carbon and

energy source. For this, 250-ml pre-autoclaved glass Schott bottles were prepared containing 45 ml ONR7a, Macondo oil to ca. 100 mg/L final concentration, and inoculated with 5 ml of washed cells. For preparation of inocula, the strains were grown in ONR7a amended with Na-pyruvate (for TK-46(2) and TK-105) or phenanthrene (for TK-8); the cell biomass was washed three times, resuspended in sterile ONR7a to 5 ml and used for inoculation. Additional incubations were set up in the same way with the exception that 85% phosphoric acid (3% final concentration) was added, or the bottles were not inoculated; these controls served to analyse for any loss of hydrocarbons due to abiotic factors. All incubations were carried out in triplicate and incubated in parallel in the dark with gentle shaking (100 rpm) and at 21°C, which is a temperature similar to that at the sea surface in the Gulf of Mexico during the time of the DwH oil spill. At the termination of the experiment (day 20), all the bottles were extracted for total petroleum hydrocarbons (TPH) and subsequent analysis for individual hydrocarbon constituents by gas chromatography/mass spectrometry (GC-MS), as detailed below.

For extraction of TPH, dichloromethane (DCM) was used at an oil/water mix to DCM ratio of 2:1. The DCM fraction was removed and the oil/water mix re-extracted an additional 3 times. The extracted oil sample was then diluted with DCM to ca. 5ml and dried using anhydrous sodium sulphate. An aliquot of known volume was removed, evaporated to dryness and weighed. The gravimetric data were used to calculate the original sample weight and the weight of oil remaining.

A known aliquot corresponding to ca. 30mg was taken from the remaining sample and transferred to a 10ml vial. An aliquot of the reference oil was weighed directly into a vial and diluted with ca. 0.3ml DCM. Squalane and 1,1'-binaphthyl were added as standards at ca. 0.5% and 0.05% by weight of the oil, respectively. A procedural blank including the standards was also prepared. One sample was analysed in triplicate and the reference oil was analysed in duplicate.

A chromatographic column was prepared using silica topped with alumina. Both sorbents were pre-extracted with DCM and activated at 120°C prior to use. The sorbents were introduced as slurries in petroleum ether (b.p. range 40-60°C). The sample (sorbed to ~3 g alumina) was applied

to the top of the column. The total petroleum hydrocarbon (TPH) fraction was eluted with 50ml petroleum ether followed by 70ml petroleum ether/DCM (2:5). Solvent was reduced to 3ml using a Heidolph rotary evaporator and an aliquot was removed for gas chromatographic analysis.

The TPH fractions were analysed on a Hewlett Packard 5890 GC fitted with a split/splitless injector (300°C), a flame ionisation detector (FID) (310°C) and an HP-5 capillary column (J&W, 30m x 0.25mm i.d. x 0.25µm film thickness). Samples were injected using a Hewlett Packard 6890 automatic injector. The oven programme was 50°C (2 min) – 5°C/min – 300°C (20 min) giving a total run time of 74 min. Chromatographic data were acquired and processed using an Atlas 8.3 Chromatographic Data System (Thermo Scientific). Peak areas for individual C_8 to C_{35} n-alkanes, the isoprenoids pristane and phytane, and for the added standard squalane were obtained. The total hydrocarbon content was calculated using the manually integrated area under the whole chromatogram, excluding the solvent peak. The corresponding total area for the procedural blank (which also contained the added standards) was then subtracted from the total area obtained for the samples and reference oil. Analyte concentrations were measured using the areas of the added standards, assuming a response factor of one, and are thus semi-quantitative. Full quantitation (using a range of standards and individual analyte response factors) was not carried out since the purpose of the experiments was a comparison different treatments including controls, using the same analytical protocols. The aromatic hydrocarbons in the TPH fractions were analysed by GC-MS on an Agilent 7890A GC fitted with a split/split less injector (at 280°C) linked to an Agilent 5975C MSD, with data acquisition and processing by Agilent Chemstation software. Selected samples were analysed in full scan mode (50-600 amu sec^{-1}) but all samples were analysed in selected ion monitoring (SIM) mode using the analyte aromatic hydrocarbon molecular ions or major fragment ions. An aliquot (1 µl) of the TPH fraction diluted in hexane/dichloromethane was injected in split/splitless mode using an Agilent 7683B autosampler and the split opened after 1 minute. Separation was performed on an Agilent fused silica capillary column (30 m x 0.25 mm i.d) coated with 0.25 µm 5% phenylmethylpolysiloxane (HP-5) phase. The GC was temperature

programmed from 50-310°C at 5°C min and held at final temperature for 10 minutes with helium as the carrier gas (flow rate of 1 ml min^{-1}, initial inlet pressure of 50 kPa, split at 30 ml min^{-1}). Individual aromatic hydrocarbon analytes were semi-quantitatively determined by comparison of their peak areas in their respective ion chromatograms with that of the added 1,1'-binaphthyl standard (m/z 253) assuming a response factor of one.

Ratios of *n*-alkanes to acyclic isoprenoid hydrocarbons (nC$_{17}$/pristane and nC$_{18}$/phytane) were used as convenient indicators of biological degradation, due to the recalcitrance imparted by the branched structure of the isoprenoid biomarkers (Sauer and Boehm, 1991; Papazova and Pavlova, 1999; Dawson et al., 2013). Similarly for aromatic hydrocarbon analysis, this was done for 7 ratios indicative of biodegradation (naphthalene/2-methylnaphthalene; 2-methylnaphthalene/1-methylnaphthalene; 2-ethylnaphthalene/2,6+2,7-dimethylnaphthalene; 2-methylnaphthalene/2,6+2,7-dimethylnaphthalene; phenanthrene/9-methylphenanthrene; 3+2-methylphenanthrene/9+1-methylphenanthrene; 3-methylphenanthrene/9-methylphenanthrene). Concentrations of aliphatic and aromatic hydrocarbon species/groups that were biodegraded after 20 days were also calculated by subtracting the respective hydrocarbon concentrations measured in the acidified controls from those of the non-acidified incubations. A Student's *t*-test was performed to test for significant differences ($P < 0.05$) in the degradation of the hydrocarbons by each strain analysed against their respective uninoculated/acid-inhibited controls.

Roller-bottle incubation experiments

The potential of strains TK-46(2), TK-105 and TK-8 in promoting the formation of MOS and/or emulsification of oil was investigated using a roller-bottle design, as described previously (Gutierrez et al. 2013a). This type of experimental setup maintains the system in a constant gentle turbulence, thereby reducing the potential of particles to settle on the container walls (Jackson, 1994) and simulates conditions near the sea surface. Autoclaved synthetic seawater, ONR7a, was utilized in these experiments to directly associate the formation of any MOS and/or emulsions to the

respective strain, as natural seawater, even if pre-sterilised, can contain substances (e.g. EPS, TEP) that can induce the formation of MOS or emulsification of crude oil.

For each strain, three roller-bottle experiments were run in duplicate. The first involved the use of inactive/non-respiring cells (Na-azide treated) to examine the potential of the cell surface alone to promote the formation of MOS or the emulsification of the oil – hereafter referred to as the azide-inhibited incubations. The second employed using cell-free fractions to determine if any extracellularly-released EPS from these strains promoted the same effect – hereafter referred to as the cell-free fraction incubations. The third experiment used live cells to evaluate whether cell activity, such as degradation of the oil by the strains, might promote MOS and/or emulsion formation – hereafter referred to as the live-cell incubations. The inoculum for each of these experiments was prepared by growing up a large batch of cells in ONR7a amended with filter-sterilized (0.2 µm) Macondo oil (0.5% v/v final concentration), glucose (0.05% w/v final concentration), and a trace elements and vitamin mixture (Dyksterhouse et al. 1995). The cell biomass was recovered by centrifugation (8,000 x g; 20 min) and the supernatant fraction set aside. The cells were washed twice with autoclaved ONR7a, re-suspended to a final optical density (600 nm) of 0.06, and supplemented with Na-azide (0.01% w/v final concentration) to render the cells inactive for use in the azide-inhibited incubations. For preparation of the cell-free fraction incubations, the above supernatant fractions for each respective strain were filtered (0.2 µm) in order to remove all residual cells and then supplemented with Na-azide as above. Washed 'live' cells (i.e. in the absence of Na-azide treatment) were used (0.06 final OD_{600}) for the live-cell incubations. Each of the three incubations was conducted in duplicate using 250-ml Pyrex© glass bottles (27 x 37 mm) which were filled with 200 ml of the respective inoculum fraction, and to each 1 ml of sterile oil was added to a final oil slick content of 0.5% (v/v). Control incubations were run in parallel using uninoculated autoclaved ONR7a in the presence or absence of oil. Inoculated controls were also included, but without the addition of oil. The bottles were incubated at 21°C in the dark on a roller table at 3.5 rpm for 14 days. The bottles were periodically placed upright to

photographically record the formation of MOS and/or emulsification of the oil. Samples were also withdrawn for light microscopy or staining with acridine orange (Francisc et al., 1973) for imaging with a FITC filter on a Zeiss Axioscope (Carl Zeiss, Germany).

At the end of these 14-day roller bottle incubations, MOS particles that formed were carefully withdrawn using glass Pasteur pipettes and transferred to 1.5-ml microtubes for staining with the cationic copper phthalocyanine dye alcian blue (AB) at pH 2.5 (Alldredge et al. 1993), or with the amino acid-specific dye coomassie brilliant blue G (CBBG) at pH 7.4 (Long and Azam, 1996). AB is used for staining acidic sugars of EPS, whereas CBBG is used for staining the proteinaceous component of EPS. Following staining, the MOS particles were washed by transferring them through several droplets of sterile water prior to their examination under the light microscope.

Production and extraction of EPS from strain TK-46(2)

Strain TK-46(2) was grown in 3-liter Erlenmeyer flasks containing 770 ml of ZM/10 medium amended with glucose (0.5% w/v) and incubated (28°C; 150 rpm) for 3 days. The cell biomass was then removed by centrifugation (10,000 $\times$g; 20 min) and filtration (0.2 μm), and the resultant cell-free supernatant treated with 7.5% (w/v) KCl and two volumes of cold absolute ethanol to precipitate the polymer. After the precipitate was allowed to settle for 24 h at 4°C, it was recovered by centrifugation (4,500 xg; 10 min) and subsequently dialysed (50 kDa MWCO) extensively over a period of 3 days against distilled water at 4°C. The dialysed material was then lyophilised and used for subsequent chemical analysis, as described below.

Chemical analysis of the TK-46(2) EPS

To determine the monosaccharide composition, triplicate samples (10 μl at 1% [wt/vol]) of polymer TK-46(2) were dissolved in 500 μl of 2 M trifluroacetic acid and hydrolysed at 100°C for 4 h (Gutierrez et al. 2008). The samples were then prepared for analysis by high-performance anion

exchange chromatography using a Dionex Carbopac PA-20 column on a Dionex ICS-3000 Ion Chromatography System (Dionex Corp. Sunnyvale, USA) and eluted with 0.01 M NaOH at a flow rate of 0.3 ml/min for 20 minutes to elute neutral sugars and then for a further 20 minutes with 1 M NaOAc in 0.15 M NaOH to elute uronic acid residues. The monosaccharide composition was then quantified using external standards. The total carbohydrate content was calculated from the individual amounts of monosaccharides.

For determination of amino acid composition, acid hydrolysis was performed on 3 mg of the TK-46(2) polymer. Samples were hydrolyzed at 110°C in 2 ml of 6M HCl for 24 h under vacuum and then dehydrated and diluted in 0.1M HCl. Analysis was performed using a Waters 2695 Separations Module, a 2487 Dual Absorbance Detector and a 1515 Isocratic high-performance liquid chromatography (HPLC) Pump equipped with a 300 x 3.5 mm Laborsevice 7-micron resin cation exchange column. Quantification was performed using a Sigma Amino Acid Standard (AAS18) external calibrant. The total protein content was calculated from the individual amounts of amino acids.

For molecular weight and polydispersity determination of polymer TK-46(2), size-exclusion chromatography coupled to multi-angle laser light scattering (SEC-MALLS) was used. For this, the polymer was dissolved in distilled water at ~0.3% (wt/vol) and then analysed by size exclusion chromatography at ambient temperature on a PL Aquagel guard column (Polymer Labs, Amherst, U.S.A.) which was linked in series with PL Aquagel-OH 60, PL Aquagel-OH 50 and PL Aquagel-OH 40 and was eluted with 0.1 M $NaNO_3$ at a flow rate of 0.7 ml/min. The eluent was then detected online firstly by a DAWN EOS light scattering detector (Wyatt Technology, Santa Barbara, U.S.A.) and by a rEX differential refractometer (Wyatt Technology, Santa Barbara, U.S.A.). The refractive index increment, *dn/dc* was taken to be that of a typical polysaccharide (0.150 ml/g) (Harding et al., 1991; Theisen et al., 2000). Samples were run in triplicate.

For ^{1}H nuclear magnetic resonance (NMR) analysis, the TK-46(2) polymer was dissolved in D_2O (to ~0.7 ml) containing 1 μl of 2% acetone in D_2O as an internal reference. Proton NMR

spectra were acquired at 60°C using a Bruker AVIII 400 MHz spectrometer. Temperature regulation utilized a BVT3200 temperature control unit. One-dimensional spectra were acquired using the Bruker pulse program 'zgesp' featuring a water-suppression sequence. The number of scans was set at 256, the acquisition time was ca. 1 second and a line-broadening factor of 1Hz was imposed on the data prior to processing. TOCSY spectra were acquired using the Bruker pulse program 'dipsi2esgpph' featuring a water-suppression sequence. TD(1) was set at 256W and the data was truncated in f_2 with TD_{eff} set to 800W, TD(2) being set to 2048W. The mixing time was set to 120ms. COSY spectra were acquired using the Bruker pulse program 'cosydfgpph19' featuring a water-suppression sequence. TD(1) was set at 256W and the data was truncated in f_2 with TD_{eff} set to 800W, TD(2) being set to 2048W.

Emulsification assays

To evaluate the potential of strains TK-46(2), TK-105 and TK-8 to produce surface-active agents such as bio-surfactants or bio-emulsifiers, they were incubated in ONR7a or ZM/100 marine medium supplemented with Macondo oil (0.05% v/v) or glucose (0 to 0.5% w/v). After 3-4 days incubation (120 rpm; 21°C), emulsification assays and surface tension measurements were performed on cell-free fractions of the cultures, as previously described (Gutierrez et al. 2007). Briefly, to assay for emulsification activity, the samples were mixed with an equal volume of the oil (Macondo oil or *n*-hexadecane) in acid-washed (0.1 N HCl) screw-cap glass tubes (100 x 13 mm), manually shaken (15 s) and vortexed (15 s) to homogeneity, left to stand for 10 min, shaken as before, and the height of the emulsion layer – expressed as Emulsification Index, EI_{24} – measured after allowing the mixture to stand for 24 h at 21°C. For tensiometry, a Kibron Ez-Pi Plus tensiometer was used with a Kibron Dyne Probe for static du Noüy measurements.

Results

Degradation of Macondo crude oil by the strains

Hydrocarbon degradation tests showed that the *Alteromonas*, *Pseudoalteromonas* and *Cycloclasticus* strains degraded distinct substrate classes in the Macondo oil. For this, the total volume of each of the cultures for each strain were extracted and analysed for total petroleum hydrocarbons (TPH) and their hydrocarbon composition, as described below. Compared with uninoculated/acid-inhibited controls, the concentrations of some hydrocarbon species were found to have significantly decreased ($P < 0.05$) after 20 days in the 'live' (no acid treated) incubations, and could thus be attributed to biodegradation by the strains. Table 1 shows these hydrocarbons that were significantly biodegraded for each of the three strains. Of a total of 28 *n*-alkanes analysed (nC$_8$–nC$_{35}$), five were significantly degraded (i.e. nC$_{12}$–nC$_{15}$) by strain TK-46(2) and another two (nC$_{14}$, nC$_{15}$) by strain TK-8 (Table 1). All other *n*-alkanes were either not biodegraded or their loss was accounted for by abiotic degradation respective to the controls. From these same experiments, ratios of *n*-alkanes to acyclic isoprenoid biomarkers, and similarly ratios for aromatic hydrocarbons, were used as indicators of biological degradation (Figure 1). For strain TK-46(2), reductions in nC$_{18}$/phy, 2-methylnaphthalene/1-methylnaphthalene and 2-methylnaphthalene/2,6+2,7-dimethylnaphthalene were measured. For strain TK-105, reductions in naphthalene/2-methylnaphthalene, 2-methylnaphthalene/1-methylnaphthalene and 2-methylnaphthalene/2,6+2,7-dimethylnaphthalene were measured. For strain TK-8, reductions in naphthalene/2-methylnaphthalene, 2-methylnaphthalene/1-methylnaphthalene, 2-methylnaphthalene/2,6+2,7-dimethylnaphthalene, phenanthrene/9-methylphenanthrene and 3-methylphenanthrene/9-methylphenanthrene were measured. No significant reductions for nC$_{17}$/pristane, 2-ethylnaphthalene/2,6+2,7-dimethylnaphthalene and 3+2-methylphenanthrene/9+1-methylphenanthrene were measured. These results, as expected, show that strain TK-8 excelled in degrading the aromatic hydrocarbon fraction of the oil compared to the other two strains. Taken collectively, the strains exhibited a capacity to degrade a wide range of hydrocarbon species, with

strain TK-8 specialising in the degradation of aromatic hydrocarbons. Higher-molecular-weight aromatic compounds, such as substituted/non-substituted dibenzothiophenes, monoaromatic steroids and triaromatic steranes (measured from their respective ion chromatogram peak areas against that of the added 1,1'-binaphthyl, using a response factor of one), did not appear to be degraded by any of the three strains, at least under the experimental conditions employed.

MOS formation and Macondo oil emulsification potential by the strains

In live-cell roller bottle incubations with *Alteromonas* strain TK-46(2) incubated with synthetic seawater medium ONR7a (Dyksterhouse et al. 1995) amended with filter-sterilized Macondo crude oil, MOS formation was observed after 5 days with the abundance of MOS aggregates increasing until day 12 (Fig. 2A). Similar observations were recorded for this strain in the azide-inhibited incubations, with the exception that MOS formation did not occur until day 7. For both the live-cell and azide-inhibited incubations, the diameter of the MOS particles formed ranged from 2 to 5 mm. They displayed a 'fluffy' off-white appearance and none of the particles settled to the bottom when the bottles were held upright – all the particles floated and localised just under the partially emulsified oil slick. Upon close inspection, they were found to contain small associated oil droplets (inset to Fig. 2A). When submerged within the bottles after applying a gentle mixing, the MOS particles floated slowly back up and settled just under the oil slick, and at no time over the duration of these 14-day incubations did they settle to the bottom of the bottles. When stained with Acridine Orange (AO) and viewed under the epifluorescence microscope, the MOS particles from both incubations treated with or without azide were found loaded with attached cells and oil droplets (Fig. 2B). When viewed under the light microscope with the aid of dark field illumination, the particles appeared as an amorphous 'cotton wool-like' material that partially stained with the polysaccharide-specific dye Alcian Blue (AB), and were seen floating in a 'sea' of emulsified oil droplets (dark brown spheres; average size range <1 to 5 µm i.d.), of which many appeared embedded within the MOS particles (Fig. 2C). The MOS particles, however, did not stain with the

protein-specific dye Coomassie Brilliant Blue G (CBBG) (Fig. 2D). Conversely, no formation of MOS or emulsification of the oil was observed in roller bottles incubated with cell-free fractions of strain TK-46(2).

In roller bottle incubations containing live cells, azide-inhibited cells, or cell-free fractions of strain *Cycloclasticus* strain TK-8 or *Pseudoalteromonas* strain TK-105, no MOS was observed to have formed at any point during these 14-day incubations. However, in incubations with TK-105, the oil had shown signs of partial emulsification by day 3, and became progressively more emulsified over the next few days until the termination of these experiments. Increasing emulsification was indicated by the presence of small oil droplets that appeared submerged just below the oil slick when the bottles were held upright and gently agitated (Fig. 2E). In none of these incubations had the oil become completely emulsified since a clear slick of non-emulsified oil remained intact at the termination of these experiments. Light microscopic examination of the emulsified oil droplets located under the oil slick did not reveal the presence of TK-105 cells associated with the oil (not shown). Conversely, oil droplets withdrawn from live-cell incubations with TK-8 and viewed under the light microscope revealed they were coated with a dense population of the cells (Fig. 2F). Oil droplets in these incubations with TK-8 were sparse, and essentially the oil slick layer did not appear emulsified in any of the roller bottle incubations that contained live cells, azide-inhibited cells or cell-free fractions of this strain. It is worthy to note that the cell-free fraction for this strain, prepared from growth of TK-8 in ONR7a amended with Macondo oil, exhibited an orange appearance, which is indicative of the extracellular accumulation of an oxidized intermediate(s) from the metabolism of aromatic hydrocarbons (e.g. PAHs) in the Macondo oil (Cuskey and Olsen, 1988).

In uninoculated control incubations with ONR7a amended with oil only, or ONR7a inoculated with either of the three strains in the absence of oil, no signs of MOS formation or emulsification of the oil were observed at any point over the course of these 14-day experiments (results not shown).

Chemical composition and molecular mass of the TK-46(2) EPS

A monosaccharide analysis of the EPS produced by *Alteromonas* sp. strain TK-46(2) showed that it contained a carbohydrate content of 39.2 ± 7.4% of the total weight of dried polymer (Table 2). The polymer was composed of hexoses (rhamnose, fucose, galactose, glucose, mannose), amino sugars (glucosamine), uronic acids (galacturonic and glucuronic acid), and the pentose xylose. Rhamnose (26.3% ± 2.7%) and galactose (47.8% ± 2.7%) were the most abundant. All other monosaccharides were each present at less than 10% and together they contributed about 25.9% ± 0.9% to the total carbohydrate content, with trace quantities of fucose detected. The total uronic acid content of the TK-46(2) EPS was 13.4%, as contributed by galacturonic and glucuronic acids.

The total amino acid content of the TK-46(2) EPS was 6.4% ± 0.3% (Table 3) of the total weight of dried polymer. Amino acid analysis of hydrolyzed samples identified the presence of four major amino acids – aspartic acid, glutamic acid, glycine, and alanine – that in total contributed 51.7% ± 0.6% to the total amino acid content. The percent contribution of hydrophobic nonpolar amino acids to the total amino acid content was 50.6%, whereas that of polar amino acids was 49.4%. Lipid analysis did not reveal any fatty acids.

Analysis of the EPS produced by *Alteromonas* sp. strain TK-46(2) by SEC-MALLS showed it composed of a molecular-weight average molar mass (M_w) of 150,000 ± 4,000 g/mol, with a peak-average molar mass (M_p) of 134,000 ± 2,000 g/mol. The polydispersity index (M_w/M_n) of the EPS was 2.75 ± 0.18. As polydispersity provides an indication of the molecular size distribution of a polymer in solution, this value for the EPS produced by strain TK-46(2) denotes that it is heterogenous compared to, for example, commercially available pullulan ($I_p \leq 1.1$). Generally, a polydispersity index of ≥ 1.6 is indicative of a polydisperse polymer (Harding et al., 1991). It should be noted that the weight-average molecular weight of a mixture of components will be biased toward higher molecular weights due to the way weight-average molecular weights are calculated. This molecular-weight heterogeneity may be expected to be higher in seawater because

the presence of salt can cause these types of marine bacterial polymers to further dissociate into smaller molecular-weight species. This behaviour can result from the high content of anionic moieties, such as uronic acids, which are typically enriched in marine bacterial EPS (Decho and Gutierrez, 2017).

Figure 3A displays the expansion of the 1D ^{1}H NMR spectrum of the TK-46(2) EPS showing strong peaks from the glycan component predominating and the typical appearance of a complex carbohydrate spectrum. There is a downfield group of at least 6 fairly well resolved anomeric signals between 4.7 and 5.5 ppm, characteristic of the α configuration at the anomeric centre. An envelope of overlapping signals between 3.3 and 4.3 ppm originates from the ring and methylene protons of sugars. A strong group of signals at about 1.3 ppm may arise from protons at rhamnose C6 (see Table 2); a peak near 2.05 ppm may include the N-acetyl methyl signal, from the small proportion of glucosamine in the sample (see Table 2). A sharp peak at 1.5 ppm may arise from residual solvent, and the acetone internal standard signal is seen at 2.22 ppm. The small proportion of amino acids in the sample (Table 3) is also reflected in the ^{1}H NMR spectrum, though signals from methyl groups of alanine and threonine coincide with the rhamnose methyl in the envelope of peaks near 1.3 ppm. The TOCSY spectrum displayed in Figure 3B shows a group of cross-peaks linking the anomeric and ring protons, and a further group of cross-peaks linking the signals assigned as H6 of rhamnose with the ring proton region. An unidentified component gives rise to a spin system of signals at about 1.6, 2.1, and 2.4 and 3.8 ppm, not characteristic of any of the sugars listed in Table 2; this group of signals may possibly be attributed to the presence of proline. The six major and several other minor signals in the anomeric region of the spectrum indicate a complex structure for the polysaccharide, with some degree of heterogeneity. This structural spectrum is comparable with exopolysaccharides from other *Alteromonas* strains (Le Costauëc et al. 2012).

Surface-active qualities of the TK-46(2) EPS

Cell-free fractions of the strains TK-46(2) and TK-105, during growth in ONR7a or ZM/100 medium amended with glucose, resulted in partial emulsification of Macondo oil or *n*-hexadecane in emulsification assays. No emulsification occurred with cell-free extracts of strain TK-8. The lyophilized TK-46(2) polymer was dissolved in seawater at a concentrations of 0.2% (w/v) and the solution was used to perform the emulsification assay and measure for any reduction to the surface tension of seawater. The polymer was found to strongly emulsify crude oil and the hydrocarbon oil *n*-hexadecane, resulting in EI_{24} values of 100%, indicating that the oil layer had been completely emulsified in these assays. Neither the lyophilized polymer nor cell-free fractions of the three strains resulted in a marked reduction to the surface tension of seawater to below 62.5 mN/m at 21°C.

Discussion

Hydrocarbon analysis revealed that all three strains (*Alteromonas* TK-46(2), *Pseudoalteromonas* TK-105 and *Cycloclasticus* TK-8) were able to degrade various aliphatic and aromatic hydrocarbons of the Macondo oil. As expected, strain TK-8 degraded a range of aromatic hydrocarbons, which is typical for the genus *Cycloclasticus* (Head et al., 2006; Yakimov et al., 2007). Notably, however, we found this strain also able to degrade aliphatics, specifically nC_{14} and nC_{15}, representing the first direct evidence for *Cyclocasticus* to have contributed to the degradation of the saturated fraction of hydrocarbons in the Macondo oil; this genus had previously been recognised to specialise in the degradation of PAHs only. Supporting this new ecophysiological role, a recent study analysing reconstructed genomes of several dominant hydrocarbon-degrading taxa that were enriched during the DwH spill showed that *Cycloclasticus* enriched on PAH substrates in stable isotope probing experiments encoded a near-complete *n*-alkane degradation pathway as well (Dombrowski et al. 2016). Another recent study also showed members of *Cycloclasticus*, in this case that were found living in symbiosis with mussels and sponges in deep-

sea gas and oil seeps, to be capable of utilising short-chain alkanes (Rubin-Blum et al., 2017). These results from mutually independent investigations indicate that polycyclic aromatic carbon sources do not represent the full substrate spectrum of *Cyclcoclasticus* (Head et al. 2006; Yakimov et al. 2007) in the environment. The ability of strain TK-8 to metabolise substituted polycyclic aromatic compounds together with *n*-alkanes strongly suggests that members of *Cycloclasticus* had contributed not just to the degradation of the aromatic hydrocarbons of the Macondo oil, but also the aliphatic fraction; though likely less significantly for the latter as species of this genus are mainly recognized for their almost exclusive preference for utilizing aromatic hydrocarbons (Head et al., 2006; Yakimov et al., 2007). Taken together, the versatility of strain TK-8 to degrade both aromatic and aliphatic hydrocarbons, its affinity for attaching to oil droplets, and the strong enrichment of *Cycloclasticus* populations during the DwH spill, identifies this genus as a major protagonist in the biodegradation of the Macondo oil.

Interestingly, our hydrocarbon analysis did not reveal strains TK-46(2) or TK-105 capable of degrading phenanthrene in the Macondo oil, yet both strains were previously shown to utilise this compound as a sole carbon source (Gutierrez et al. 2013b). Enrichments with complex hydrocarbon mixtures, such as crude oil, can result in competitive inhibition (Stringfellow and Aitken, 1995), and could explain why both strains did not show significant degradation of phenanthrene in the Macondo oil, even though their ability to utilise this compound has been substantiated in pure culture experiments (Gutierrez et al. 2013b). Furthermore, whilst strain TK-105 was not identified to significantly degrade any n-alkane species, it is possible that this 'generalist' hydrocarbon degrader may encode the capacity to do so, but this was not possible due to competitive inhibition effects.

Species of *Pseudoalteromonas* and *Alteromonas* are important members of the 'generalist' hydrocarbon-degrading community in marine environments, and these organisms were enriched during the DwH spill by at least one order of magnitude compared to their abundance in uncontaminated reference waters (Gutierrez et al., 2013a); they have also been found abundant in

natural hydrocarbon seep sites in the Gulf of Mexico (Kleindienst et al. 2016). Considering the confirmed hydrocarbon-degrading ability of *Alteromonas* TK-46(2) and *Pseudoalteromonas* TK-105, these organisms, together with *Cycloclasticus*, had likely contributed significantly to the degradation of the Macondo oil in sea surface oil slicks where they became strongly enriched (Hazen et al., 2010; Gutierrez et al., 2013b; Valentine et al., 2010). It is possible that these *Pseudoalteromonas* and *Alteromonas* strains contributed here in part through their known ability to produce EPS exhibiting surface-active properties (i.e. acting as bioemulsifiers or biosurfactants), which in turn are known to enhance the dispersion and aqueous solubility of hydrocarbons (Bozzi *et al.*, 1996; Cambon-Bonavita *et al.*, 2002; Colliec Jouault *et al.*, 2001; Gutierrez *et al.*, 2007; Gutierrez *et al.*, 2008; Mancuso Nichols *et al.*, 2004; Marx *et al.*, 2009; Qin *et al.*, 2007; Raguenes *et al.*, 1996). This enhanced-dissolution effect may have consequently promoted the degradation of hydrocarbons by these and other bacteria like the dominant *Cycloclasticus*. As the emulsification of crude oil is a natural weathering process, the *de novo* synthesis of surface-active EPS by these strains, in particular TK-46(2) and TK-105, may have contributed to the observed emulsification of the crude oil slick in these bottle experiments, albeit at varying levels of emulsification.

Whilst not recognised for producing EPS, the strong enrichment of *Cycloclasticus* in sea surface oil slicks during the DwH spill may have been influenced by the surface-active properties of EPS produced by the EPS-producers *Alteromonas*, *Pseudoalteromonas*, and the previously described *Halomonas* sp. strain TGOS-10 (and other halomonads) that was also enriched in sea surface oil slicks (Gutierrez et al., 2013a). Our analysis of the carbohydrate component of the TK-46(2) EPS revealed it to have a relatively high uronic acid content of 13.4%. The carboxylate and methoxycarbonyl groups of these types of acidic sugars can mediate the adsorption of EPS to oil droplets and form stable emulsions (Dea and Madden 1986; Kaplan et al. 1987; Tolstogusov 1991, 1994), which may explain the oil emulsions that formed in roller-bottle incubations with live and azide-inhibited cells of strain TK-46(2) and TK-105. The presence also of 6-deoxyhexoses (e.g. rhamnose) and increased substitution by acetyl moieties (NMR peak near 2.05 ppm) on the TK-

46(2) EPS can also render these types of polymers with surface-active qualities (Dea and Madden 1986; Graber et al. 1988).

Conjecture still surrounds the mechanism underlying MOS formation at DwH, as well as during the Ixtoc-I (Boehm and Fiest, 1980; Jernelöv and Lindén, 1981; Patton et al., 1981) and *Tsesis* (Johansson et al., 1980) oil spills where its formation has been proposed (Teal and Howarth, 1984). Several reports provide evidence to implicate bacterial EPS in its formation (Gutierrez et al., 2013a; Passow *et al.*, 2012; Ziervogel *et al.*, 2012), possibly via the physicochemical interaction between oil droplets and bacterial EPS (Bælum *et al.*, 2012; Passow *et al.*, 2012). Corroborating with the results from these studies, our roller-bottle experiments with *Alteromonas* sp. strain TK-46(2) showed that the EPS it produced played a role in MOS formation, and that the presence of bacterial cells (in our case with strain TK-46(2)) was required also for this process to occur (Gutierrez et al., 2013a; this study) since incubations with EPS alone (i.e. cell-free fractions) did not yield MOS. Further supporting the role for strain TK-46(2) and its EPS in the formation of MOS during the early phase of the DwH spill, we isolated a *Alteromonas* strain (matching strain TK-46(2) with 100% 16S rRNA gene sequence identity) from a MOS particle that formed in roller-bottle incubations with sea surface water collected during the active phase of the DwH spill (unpublished data). Distinct bacterial phylotypes found on MOS particles compared to those in the surrounding seawater have been observed in experiments assessing MOS formation in surface waters of the Gulf of Mexico (Arnosti et al., 2016) and of the northeast Atlantic (Duran Suja et al., 2017). In these studies, MOS particles were found enriched with various different genera of bacteria, including EPS-producers such as *Halomonas*, *Pseudoalteromonas* and *Alteromonas*, corroborating our results that showed MOS particles heavily colonized with cells of strain TK-46(2).

The surface-active (amphiphilic) properties of the TK-46(2) polymer indicates it may have played a role in the dissolution of the oil during the spill and in enhancing the bioavailability of hydrocarbons for microbial degradation. Collectively, these results provide evidence that EPS-

producing bacteria, such as *Alteromonas* sp. strain TK-46(2), contributed to the formation of MOS during the DwH spill. However, not all EPS-producing bacteria, at least not when acting alone, lead to MOS formation, as evidenced in the roller bottle incubations with *Pseudoalteromonas* sp. strain TK-105. This raises important questions that aim to understand the influence of chemical composition and surface chemistry of these types of biopolymers, including that of bacterial and other types of microbial cell surfaces, in forming MOS. Interestingly, the MOS particles that formed remained floating and did not settle to the bottom of the roller bottles over the 14-day duration of the experiments. The chemical nature of MOS particles, their microbial composition and levels of oil entrainment will influence whether they settle and the timing of settling.

A major knowledge gap exists in our understanding on the biodegradation of crude oil associated with MOS, and specifically on whether biodegradation rates are higher on MOS particles than in the surrounding seawater environment. Does MOS, when formed, enhance the biodegradation of crude oil in the water column from anytime it is formed until it subsequently settles to the seafloor? Heightened levels of hydrolytic enzyme activities, including lipases, were reported associated with MOS particles that formed in roller-bottle incubations containing sea surface water collected during the early phase of the DwH spill (Ziervogel et al., 2012), thus suggesting this might be the case. Hitherto, only a few studies have analysed and compared the microbial communities associated with MOS to that in the surrounding seawater, and their results appear to suggest that MOS may indeed act as a hotspot for microbial oil-biodegradation activity (Arnosti et al. 2016; Duran Suja et al., 2017). Compared to the surrounding seawater, MOS particles were shown to harbour higher abundances of recognised hydrocarbon-degrading (obligate and generalist hydrocarbonoclastic) bacteria, such as *Cycloclasticus*, *Halomonas*, *Marinobacter* and members of the *Roseobacter* clade associated with MOS formed in Gulf of Mexico surface waters collected during the DwH spill (Arnosti et al., 2016; Giebel et al. 2016), and *Alcanivorax*, *Pseudoalteromonas*, *Alteromonas*, *Halomonas*, *Vibrio*, *Thalassospira*, *Thalassolituus*, *Cycloclasticus* and *Marinobacter* associated with MOS formed in waters from a subarctic northeast

Atlantic surface seawater environment (Duran Suja et al., 2017). The 16S rRNA gene sequence of strain TK-8 was a 100% and 98% match to that of *Cycloclasticus* clones Oil-BE-081 and Oil-BE-051, respectively, that were identified enriched in MOS particles reported by Arnosti et al. (2016), thus implicating this organism in playing a pivotal role in the biodegradation of MOS-associated oil during the spill. Supporting this, *Cycloclasticus* was also found in high abundance in freshly-deposited seafloor sediments in the Gulf following the DwH spill (Yang et al. 2016b). The enrichment of oil-degrading bacteria in MOS is important for understanding the fate of crude oil in the event of an oil spill at sea and warrants further investigation.

Positive staining of the MOS particles with AB, and not CBBG, provided evidence of their composition as predominantly polysaccharide. This agrees with chemical characterization, using monosaccharide, amino acid and proton NMR analyses, of the TK-46(2) EPS, which will have dominated the composition of the total polymeric matter that comprised the MOS particles. The low protein content (6.4% of total polymer; Table 3) of the EPS produced by this strain is consistent with that of other marine *Alteromonas* species (Rougeaux et al., 1996; Cambon-Bonavita et al., 2002; Raguenes et al., 2003), as also with the chemical composition of marine EPS, which makes up much of the dissolved organic matter (DOM) in the ocean and is largely derived from phytoplankton and bacterial exudates (Gutierrez et al., 2007; Hassler et al., 2011; Mancuso Nichols et al., 2004). Glucose is the most abundant simple sugar in the ocean, with concentrations averaging from 0.001 to 1.0 µmol/L (Benner, 2002; Skoog et al, 2002; Rich et al, 1996, 1997), and as high as 187 nM measured in unfiltered water of the Gulf of Mexico (Skoog and Benner, 1997). Hence, a proportion of the DOM, as EPS macromolecules, in surface waters of the Gulf of Mexico prior to the onset of the DwH oil spill may likely have been the product from the metabolism of this endogenous source of glucose by EPS-producing bacteria, like *Alteromonas*, *Pseudoalteromonas* and *Halomonas*, that were present in surface waters of the Gulf of Mexico (Gutierrez et al., 2013a,b; Yang et al., 2016a). A study by Eenennaam et al. (2016) showed that EPS released by bacteria, albeit associated with phytoplankton, played a more dominant role in MOS formation than

EPS produced by axenic phytoplankton or free-living (non-associated) bacteria. Hence, we posit that bacterial-derived exopolymers may have contributed a more dominant role in MOS formation during the DwH spill than exopolymers from other sources.

Our findings reveal that dominant members of *Cycloclasticus*, and the EPS-producers *Alteromonas* and *Pseudoalteromonas*, that were enriched in sea surface oil slicks during the DwH oil spill (Gutierrez et al., 2013b; Yang et al., 2016a), were capable of degrading a range of aromatic and aliphatic hydrocarbons of the Macondo oil. Since experiments conducted in the laboratory will not represent the 'true' environmental conditions of the Gulf of Mexico during the spill, the hydrocarbon-degrading capabilities of these strains would be a snapshot of their potential in this respect. Based on their enrichment during the spill and hydrocarbon-degrading capabilities, these organisms likely contributed significantly to the biodegradation of the oil, which was possibly enhanced via the production of EPS, as previously reported for *Halomonas* enriched during the spill (Gutierrez et al., 2013a). Notably, in the presence of Macondo oil, cells of *Alteromonas* sp. strain TK-46(2) and its produced EPS resulted in the formation of MOS. The importance of these two key components (i.e. bacterial cells and EPS) in forming MOS is highlighted by the fact that they were a major component of the MOS particles, as revealed by chemical analysis and differential staining. Whilst more work is needed to better understand and underpin the role of MOS in the fate of the oil, evidence from this and other studies suggests that MOS may be a hotspot where oil biodegradation occurs at elevated levels in the water column. Finally, the surface-active properties of the EPS produced by these *Alteromonas*, *Pseudoalteromonas* and other oil-degrading strains (e.g. *Halomonas* sp. strain TGOS-10) (Gutierrez et al., 2013a) could be explored to develop a bioremediation agent or bio-based dispersant formulation to treat oil spills.

References

1. Alldredge, A.L., Passow, U., Logan, B.E. (1993) The abundance and significance of a class of large, transparent organic particles in the ocean. Deep-Sea Res 40: 1131-1140.

2. Arnosti, C., Ziervogel, K., Yang, T., Teske, A. (2016) Oil-derived marine aggregates – hot spots of polysaccharide degradation by specialized bacterial communities. Deep-Sea Res. II, 129: 179-186.

3. Azam, F. (1998) Microbial control of oceanic carbon flux: the plot thickens. Science, 280: 694-696.

4. Bælum, J. *et al.* (2012) Deep-sea bacteria enriched by oil and dispersant from the Deepwater Horizon spill. *Environ. Microbiol.* 14, 2405–2416.

5. Benner, R. (2002). Chemical composition and reactivity. In D.A. Hansell and C.A. Carslon (eds.) *Biogeochemistry of Marine Dissolved Organic Matter*. Academic Press, pp. 59-90.

6. Boehm, P.D., and Fiest, D.L. (1980). Aspects of the transport of petroleum hydrocarbons to the offshore benthos during the Ixtoc-I blowout in the Bay of Campeche. In: *Proceedings of the Symposium on the Preliminary Results from the September, 1979 Pierce/Research*

IXTOC-1Cruises. Key Biscayne, Florida, June 9-10, 1980. Publications Office, NOAA/RD/MP3, Office of Marine Pollution Assessment, NOAA, US Dept. of Commerce, 325 Broadway, Boulder, CO 80303, USA.

7. Bozzi, L., Milas, M., Rinaudo, M. (1996) Characterization and solution properties of a new exopolysaccharide excreted by the bacterium *Alteromonas* sp. strain 1644. Int. J. Biol. Macromol. 18: 9-17.

8. Brooks, G.R., Larson, R.A., Schwing, P.T., Romero, I., Moore, C., Reichart, G.-J., Jilbert, T., Chanton, J.P., Hastings, D.W., Overholt, W.A., Marks, K.P., Kostka, J.E., Holmes, C.W., Hollander, D. (2015) Sedimentation pulse in the NE Gulf of Mexico following the 2010 DWH blowout. PLOS one, 24 pp. doi:http://dx.doi.org/10.1371/journal.pone.0132341

9. Cambon-Bonavita, M. A., Raguenes, G., Jean, J., Vincent, P., Guezennec, J. (2002) A novel polymer produced by a bacterium isolated from a deep-sea hydrothermal vent polychaete annelid. J. Appl. Microbiol. 93:310–315.

10. Camilli R, Reddy CM, Yoerger DR, Van Mooy BAS, Jakuba MV, Kinsey JC, et al. (2010) Tracking hydrocarbon plume transport and biodegradation at Deepwater Horizon. Science 330: 201–204. doi: 10.1126/science.1195223. pmid:20724584

11. Chanton, J., Zhao, T., Rosenheim, B.E., Joye, S.B., Bosman, S., Brunner, C., Yeager, M., Diercks, A.R., Hollander, D. (2015) Using natural abundance radiocarbon to trace the flux of petrocarbon to the seafloor following the Deepwater Horizon oil spill. Environmental Science and Technology 49: 847-854.

12. Colliec Jouault, S., Chevolot, L., Helley, D., Ratiskol, J., Bros, A., Sinquin, C., Roger, O., Fischer, A-M. (2001) Characterization, chemical modifications and in vitro anticoagulant properties of an exopolysaccharide produced by *Alteromonas infernus*. BBA, 1528: 141-151.

13. Cuskey, S.M., Olsen, R.H. (1988) Catabolism of aromatic biogenic amines by *Pseudomonas aeruginosa* PAO1 via meta cleavage of homoprotocatechuic acid. J. Bacteriol. 170:393–399.

14. Daly, K.L., Passow, U., Chanton, J., Hollander, D. (2016) Assessing the impacts of oil-associated marine snow formation and sedimentation during and after the Deepwater Horizon oil spill. Anthropocene, 13: 18-33.

15. Dawson, K.S., Schaperdoth, I., Freeman, K.H., and Macalady, J.L. (2013) Anaerobic biodegradation of the isoprenoid biomarkers pristane and phytane. Organic Geochem 65:118–126.

16. Dea, I.C.M. and Madden, J.K. (1986) Acetylated pectic polysaccharides of sugar beet. Food Hydrocolloids 1, 71–88.

17. Decho, A.W., Gutierrez, T. (2017) Microbial extracellular polymeric substances (EPSs) in ocean systems. *Frontiers in Microbiology*, 8:922. doi: 10.3389/fmicb.2017.00922

18. Diercks A-R, Highsmith RC, Asper VL, Joung DJ, Zhou Z, Guo L et al. (2010). Characterization of subsurface polycyclic aromatic hydrocarbons at the Deepwater Horizon wellhead site. Geophys Res Lett 37: L20602.

19. Dombrowski, N., Donaho, J.A., Gutierrez, T., Seitz, K.W., Teske, A.P., Baker, B.J. (2016) Reconstructing metabolic pathways of hydrocarbon-degrading bacteria from the Deepwater Horizon oil spill. *Nature Microbiology*, doi:10.1038/nmicrobiol.2016.57

20. Du, M., Kessler, J.D. (2012) Assessment of the spatial and temporal variability of bulk hydrocarbon respiration following the Deepwater Horizon oil spill. Environ Sci Technol, 46: 10499-10507.

21. Duran Suja, L., Summers, S., Gutierrez, T. (2017) Role of EPS, dispersant and nutrients on the microbial response and MOS formation in the subarctic northeast Atlantic. *Frontiers in Microbiology*, 8:676. doi: 10:3389/fmicb.2017.00676.

22. Dyksterhouse, S.E., Gray, J.P., Herwig, R.P., Cano Lara, J., Staley, J.T. (1995) *Cycloclasticus pugetii* gen. nov., sp.nov., an aromatic hydrocarbon-degrading bacterium from marine sediments. Int J Syst Bacteriol 45: 116-123.

23. Francisc DE, Mah RA, Rabin AC (1973) Acridine orange-epifluorescence technique for counting bacteria in natural waters. Trans Am Microsc Soc 92: 416-421.

24. Fu, J., Gong, Y., Zhao, X., Reilly, S.E., Zhao, D. (2014) Effects of oil and dispersant on formation of marine oil snow and transport of oil hydrocarbons. Environ. Sci. Technol. 48, 14392–14399.

25. Giebel, H.A., Klotz, F., Voget, S., Poehlein, A., Grosser, K., Teske, A., Brinkhoff, T. (2016) Draft genome sequence of the marine *Rhodobacteraceae* strain O3.65, cultivated from oil-polluted seawater of the Deepwater Horizon oil spill. Standards in Genomic Sciences 11:81, doi: 10.1186/s407893-016-0201-7

26. Graber, M., Morin, A., Duchiron, F. and Monsan, P.F. (1988) Microbial polysaccharides containing 6-deoxysugars. Enzyme Microb Technol 10, 198–206.

27. Gutierrez T, Mulloy B, Bavington C, Black K, Green DH (2007) Partial purification and chemical characterization of a glycoprotein (putative hydrocolloid) emulsifier produced by a marine bacterium *Antarctobacter*. Appl Microbiol Biotechnol 76: 1017-1026.

28. Gutierrez, T., Shimmield, T., Haidon, C., Black, K. and Green, D.H. (2008) Emulsifying and metal ion binding activity of a glycoprotein exopolymer produced by *Pseudoalteromonas* species TG12. *Applied and Environmental Microbiology*, 74: 4867-4876.

29. Gutierrez, T., Berry, D., Yang, T., Mishamandani, S., McKay, L., Teske, A., Aitken, M. (2013a) Role of bacterial exopolysaccharides (EPS) in the fate of the oil released during the *Deepwater Horizon* oil spill. *PLoS ONE*, doi: 10.1371/journal.pone.0067717.

30. Gutierrez, T., Singleton, D.R., Berry, D., Yang, T., Aitken, M.D., Teske, A. (2013b) Hydrocarbon-degrading bacteria enriched by the *Deepwater Horizon* oil spill identified by cultivation and DNA-SIP. *The ISME Journal*, 7:2091-2104.

31. Hassler CS, Alasonati E, Mancuso Nichols CA, Slaveykova VI (2011) Exopolysaccharides produced by bacteria isolated from the pelagic Southern Ocean – Role of Fe binding, chemical reactivity, and bioavailability. Mar Chem 123: 88-98.

32. Harding, S.E., Vårum, K.M., Stokke, B.T., Smidsrød, O. (1991) Molecular weight determination of polysaccharides. In: White CA, editor. Advances in carbohydrate analysis, Vol. 1. Greenwich, USA: JAI Press Ltd. pp. 63–144.

33. Hazen, T.C., Dubinsky, E.A., DeSantis, T.Z., Andersen, G.L., Piceno, Y.M., et al. (2010) Deep-sea oil plume enriches indigenous oil-degrading bacteria. Sci 330: 204-208.

34. Head, I.M., Jones, D.M., Röling, W.F. (2006) Marine microorganisms make a meal of oil. Nat Rev Microbiol 4: 173–182.

35. Hollander, DJ, Flower, BP, Larson, R, Brooks, G, Romero, I, Zinzola, N, et al. (2012) Deposition, distribution and fate of Macondo oil in the sediments of the northeastern Gulf of Mexico. 2012 Ocean Sciences Meeting. Feb 23 2012.

36. Jackson, G.A. (1994) Particle trajectories on a rotating cylinder: implications for aggregation incubations. Deep-Sea Res 41: 429-437.

37. Jernelöv, A., and Lindén, O. (1981). Ixtoc I: a case study of the world's largest oil spill. *AMBIO* 10: 299–306.

38. Johansson, S., Larsson, U., and Boehm, P. (1980). The *Tsesis* oil spill impact on the pelagic ecosystem. *Mar. Pollut. Bull*. 11, 284–293.

39. Joye SB, Teske AP, Kostka JE, (2014) Microbial dynamics following the Macondo oil well blowout across Gulf of Mexico environments. BioScience 64: 766–777. doi: 10.1093/biosci/biu121

40. Joye SB, MacDonald IR, Leifer I, Asper V. (2011) Magnitude and oxidation potential of hydrocarbon gases released from the BP oil well blowout. Nat. Geosci. 4: 160–164. doi: 10.1038/ngeo1067

41. Kaplan, N., Zosim, Z. and Rosenberg, E. (1987) Reconstitution of emulsifying activity of *Acinetobacter calcoaceticus* BD4 emulsan by using pure polysaccharide and protein. Appl. Environ. Microbiol. 53, 440–446.

42. Kleindienst, S., Grim, S., Sogin, M., Bracco, A., Crespo-Medina, M., Joye, S.B. (2016) Diverse, rare microbial taxa responded to the *Deepwater Horizon* deep-sea hydrocarbon plume. The ISME J, 10: 400-415.

43. Le Costaouëc, T., Cérantola, D., Ropartz, D., Ratiskol, J., Sinquin, C., Colliec-Jouault, S., Boisset, C. (2012) Structural data on a bacterial exopolysaccharide produced by a deep-sea *Alteromonas macleodii* strain. Carbohydrate Polymers 90: 49-59.

44. Long, R. A., and Azam, F. (1996) Abundant protein-containing particles in the sea. Aquat. Microb. Ecol. 10, 213–221. doi: 10.3354/ame010213

45. Lubchenco, J., McNutt, M. K., Dreyfus, G., Murawski, S. A., Kennedy, D. M., Anastas, P. T., Chu, S., Hunter, T. (2012) Science in support of the *Deepwater Horizon* response. Proceedings of the National Academy of Science USA, 109: 20212-20221.

46. Mancuso Nichols C, Garon S, Bowman JP, Raguenes G, Guesennec J (2004) Production of exopolysaccharides by Antarctic marine bacterial isolates. J Appl Microbiol 96: 1057-1066.

47. Marx, J.G., Carpenter, S.D., Deming, J.W. (2009) Production of cryoprotectant extracellular polymeric substances (EPS) by the marine psychrophilic bacterium *Colwellia psychrerythraea* strain 34H under extreme conditions. Can. J. Microbiol. 55, 63–72. doi: 10.1139/W08-130

48. Montagna, P.A., Baguley, J.G., Cooksey, C., Hartwell, I., Hyde, L.J., Hyland, J.L., Kalke, R. D., Kracker, L.M., Reuscher, M., Rhodes, A.C.E. (2013) Deep-sea benthic footprint of the Deepwater Horizon blowout. PLoS One 8 (8), e70540. doi:http://dx.doi. org/10.1371/journal.pone.0070540

49. Niu H, Li Z, Lee K, Kepkay P, Mullin JV (2011) Modelling the transport of oil-mineral-aggregates (OMAs) in the marine environment and assessment of their potential risks. Environ Model Assess 16: 61-75.

50. Papazova, D., and Pavlova, A. (1999) Development of a simple gas chromatographic method for differentiation of spilled oils. J Chromatogr Sci 37: 1–4.

51. Passow U, Ziervogel K, Aper V, Diercks A. (2012) Marine snow formation in the aftermath of the Deepwater Horizon oil spill in the Gulf of Mexico. Environ. Res. Lett. 7: 035301. doi: 10.1088/1748-9326/7/3/035301

52. Passow, U. (2016) Formation of rapidly–sinking, oil-associated marine snow. Deep-Sea Research II, 129: 232-240.

53. Patton, J., Rigler, M., Boehm, P., Fiest, D. (1981) Ixtoc 1 oil spill: flaking of surface mousse in the Gulf of Mexico. Nature, 290:235-238.

54. Qin, K., Zhu, L., Chen, L., Wang, P.G., Zhang, Y. (2007) Structural characterization and ecological roles of a novel exopolysaccharide from the deep-sea psychrotolerant bacterium *Pseudoalteromonas* sp. SM9913. Microbiol 153: 1566-1572.

55. Raguenes, G., Cambon-Bonavita, M. A., Lohier, J. F., Boisset, C., Guezennec, J. (2003) A novel, highly viscous polysaccharide excreted by an *Alteromonas* isolated from a deep-sea hydrothermal vent shrimp. Curr. Microbiol. 46:448–452.

56. Raguenes, G., Pignet, P., Gauthier, G., Peres, A., Christen, R., et al. (1996) Description of a new polymer-secreting bacterium from a deep-sea hydrothermal vent, *Alteromonas macleodii* subsp. *fijiensis*, and preliminary characterization of the polymer. Appl Environ Microbiol 62: 67-73.

57. Rich, J.H., Ducklow, H.W., and Kirchman, D.L. (1996). Concentrations and uptake of neutral monosaccharides along 140°W in the Equatorial Pacific: Contribution of glucose to heterotrophic bacterial activity and the POM flux. *Limnol. Oceanogr*. 41, 595-604.

58. Rich, J., Gosselin, M., Sherr, E., Sherr, B., and Kirchman, D.L. (1997). High bacterial production, uptake and concentrations of dissolved organic matter in the Central Arctic Ocean. *Deep-Sea Res. II* 44, 1645-1663.

59. Romero, I.C., P.T. Schwing, G.R. Brooks, R.A. Larson, D.W. Hastings, G. Ellis, E.A. Goddard, D.J. Hollander. (2015) Hydrocarbons in deep-sea sediments following the 2010

Deepwater Horizon blowout in the Northeast Gulf of Mexico. PLOS one, 23 pp., doi:http://dx.doi.org/10.1371/journal.pone.0128371

60.   Romero, I.C., Toro-farmer, G., Diercks, A., Schwing, P., Muller-Karger, F., Murawski, S., Hollander, D.J. (2017) Large-scale deposition of weathered oil in the Gulf of Mexico following a deep-water oil spill. Environ. Poll. 228: 179–189.

61.   Rougeaux, H., Pichon, R., Kervarec, N., Raguenes, G. H. C., Guezennec, J. G. (1996) Novel bacterial exopolysaccharides from deep-sea hydrothermal vents. Carbohydr. Polymers 31:237–242.

62.   Rubin-Blum, M., Antony, C.P., Borowski, C., Sayavedra, L., Pape, T., Sahling, H., Bohrmann, G., Kleiner, M., Redmond, M.C., Valentine, D.L., Dubilier, N. (2017) Short-chain alkanes fuel mussel and sponge *Cycloclasticus* symbionts from deep-sea gas and oil seeps. Nat Microbiol 2:17093

63.   Ryerson, T.B., Camilli, R., Kessler, J.D., Kujawinski, E.B., Reddy, C.M., Valentine, D.L., Atlas, E., Blake, D.R., de Gouw, J., Meinardi, S., Parrish, D.D., Peischl, J., Seewald, J.S., Warneke, C. (2012) Chemical data quantify Deepwater Horizon hydrocarbon flow rate and environmental distribution. Proc. Natl. Acad. Sci. USA, 109: 20246-20253.

64.   Sauer, T., and Boehm, P. (1991) The use of defensible analytical chemical measurements for oil spill natural resource damage assessment. Int Oil Spill Conf Proc, 363–369.

65.   Skoog, A., Benner, R. (1997) Aldoses in various size fractions of marine organic matter: Implications for carbon cycling, *Limnol. Oceanogr.*, 42, 1803-1813.

66.   Skoog, A., Whitehead, K., Sperling, F., and Junge, K. (2002). Microbial glucose uptake and growth along a horizontal nutrient gradient in the North Pacific. *Limnol. Oceanogr.* 47, 1676-1683.

67.   Stout, S.A., Rouhani, S., Liu, B., Oehrig, J., Ricker, R.W., Baker, G., Lewis, C. (2017) Assessing the footprint and volume of oil deposited in deep-sea sediments following the Deepwater Horizon oil spill. Mar Poll Bull 114: 327-342.

68. Stringfellow, W.T., Aitken, M.D. (1995) Competitive metabolism of naphthalene, methylnaphthalenes, and fluorene by phenanthrene-degrading pseudomonads. Appl. Environ. Microbiol. 61:357–362.

69. Teal, J.M., and Howarth, R.W. (1984). Oil spill studies: a review of ecological effects. *Environ. Manage*. 8, 27–44.

70. Theisen, A., Johann, C., Deacon, M.P., Harding, S.E. (2000) Refractive increment data book. Nottingham, UK: Nottingham University Press.

71. Tolstogusov, V.B. (1991) Functional properties of food proteins and the role of protein-polysaccharide interaction. Food Hydrocolloids 4, 429–468.

72. Tolstogusov, V.B. (1994) Some physico-chemical aspects of protein processing in foods. In Gums and Stabilizers for the Food Industry ed. Phillips, G.O., Williams, P.A. and Wedlock, D.J. pp. 115–124. Oxford: IRL Press.

73. Valentine DL, Kesser JD, Redmond MC, Mendes SD, Heintz MB, Farwell C, et al. (2010) Propane respiration jump-starts microbial response to Deep Oil spill. Science 330: 208–211. doi: 10.1126/science.1196830. pmid:20847236

74. Valentine, D.L., Fisher, G.B., Bagby, S.C., Nelson, R.K., Reddy, C.M., Sylva, S.P., Woo, M. A. (2014) Fallout plume of submerged oil from Deepwater Horizon. Proc. Natl. Acad. Sci. U.S.A. 111: 15906–15911.

75. van Eenennaam, J.S., Wei, Y., Grolle, K.C.F., Foekema, E.M., Murk, AT.J. (2016) Oil spill dispersants induce formation of marine snow by phytoplankton-associated bacteria. Marine Pollution Bulletin, 104: 294-302.

76. Verdugo, P., Alldredge, A.L., Azam, F., Kirchman, D.L., Passow, U., Santschi, P. (2004) The oceanic gel phase: a bridge in the DOM-POM continuum, Mar. Chem., 92: 67-85.

77. Vonk, S.M., Hollander, D.J., Murk, A.J. (2015) Was the extreme and wide-spread marine oil-snow sedimentation and flocculent accumulation (MOSSFA) event during the Deepwater Horizon blow-out unique? Mar. Pollut. Bull. 100: 5–12.

78. White, H.K., Hsing, P.-Y., Cho, W., Shank, T.M., Cordes, E.E., Quattrini, A.M., Nelson, R. K., Camilli, R., Demopoulos, A.W.J., German, C.R., Brooks, J.M., Roberts, H.H., Shedd, W., Reddy, C.M., Fisher, C.R. (2012) Impact of the Deepwater Horizon oil spill on a deep-water coral community in the Gulf of Mexico. Proc. Natl. Acad. Sci. USA, 109: 20303–20308.

79. Yakimov, M.M., Timmis, K.N., Golyshin, P.N. (2007) Obligate oil-degrading marine bacteria. Curr Opin Biotechnol 18: 257–266.

80. Yang, T., L. M. Nigro, T. Gutierrez, L. D'Ambrosio, S. B. Joye, R. Highsmith, A. Teske. (2016a) Pulsed blooms and persistent oil-degrading bacterial populations in the water column during and after the Deepwater Horizon blowout. Deep-Sea Research Part II 129:282-291.

81. Yang, T., K. Speare, L. McKay, B.J. MacGregor, S.B. Joye, and A. Teske. (2016b) Distinct bacterial communities in surficial seafloor sediments following the 2010 Deepwater Horizon blowout. Frontiers in Microbiology 7:1384, doi: 10.3389/fmicb.2016.01384

82. Ziervogel, K., McKay, L., Rhodes, B., Osburn, C.L., Dickson-Brown, J., Arnosti, C., Teske, A. (2012) Microbial activities and dissolved organic matter dynamics in oil-contaminated surface seawater from the Deepwater Horizon oil spill site. PLoS ONE, 7 e34816.

Figure legends

Figure 1. Differences in hydrocarbon ratios (significant results only; Student's *t*-test, $P < 0.05$) comparing the hydrocarbon-degrading capabilities of *Alteromonas* sp. strain TK-46(2), *Pseudoalteromonas* sp. strain TK-105 and *Cycloclasticus* sp. strain TK-8 to uninoculated controls for six characteristic parameters indicative of biodegradation: nC_{18}/phytane (nC_{18}/phy), naphthalene/2-methylnaphthalene (N/2-MN), 2-methylnaphthalene/1-methylnaphthalene (2-MN/1-MN), 2-methylnaphthalene/(2,6+2,7)-dimethylnaphthalene (2-MN/2,6+2,7-DMN), phenanthrene/9-methylphenanthrene (P/9-MP), 3-methylphenanthrene/9-methylphenanthrene (3-MP/9-MP). Values are averages of triplicate incubations. Error bars show standard error.

Figure 2. Formation of marine oil snow (MOS) and/or emulsions by *Alteromonas* sp. strain TK-46(2), *Pseudoalteromonas* sp. strain TK-105 and *Cycloclasticus* sp. strain TK-8 in roller bottle incubations. MOS formed in roller bottle incubations with live cells of strain TK-46(2) at day 7 shown floating underside the oil (A); *inset*, a magnified image of a MOS particle showing associated oil droplets indicated by arrows. A MOS particle formed by live cells of strain TK-46(2) viewed under the epifluorescence microscope (staining with acridine orange) shows these particles to be foci where the cells (small green dots) and oil droplets (green blobs) are found concentrated (B). Under the light microscope, these TK46(2)-formed MOS particles stained with Alcian Blue (C), but not with Coomassie Brilliant Blue (D), and were found floating in a sea of oil droplets (brown spheres), of which many were observed embedded within the amorphous matrix of the particles. Partial emulsification of the oil into small oil droplets (0.5-2.0 mm) was observed in incubations with live cells of *Pseudoalteromonas* TK105 (E). In live incubations with strain TK-8, oil droplets were found heavily coated with the cells (F). Scale bars are 5 mm in A, E; 10 μm in B, C, D, F.

Figure 3. Expansion 6.5 – 0.5 ppm of the ^{1}H NMR spectrum (400 MHz, 60 ºC in D_2O) of the TK-46(2) polymer. Strong signals arising from monosaccharide residues are labelled as arising from anomeric, ring, methylene and rhamnose methyl H6 protons. The residual partially deuterated water signal (HOD) is at about 4.4 ppm and the acetone reference signal at 2.22 ppm. Peaks marked with an asterisk are likely to arise from amino acid components, Asp, Glu and possibly Pro. B) Expansion of the TOCSY spectrum of the same polysaccharide. Boxes indicate i) cross-peaks between anomeric and ring protons of monosaccharide residues; ii) rhamnose H6 to ring proton cross-peaks; iii) cross-peaks maybe attributable to proline.

Table 1. Hydrocarbons that were significantly ($P < 0.05$) biodegraded during enrichment of strains *Alteromonas* sp. strain TK-46(2), *Pseudoalteromonas* sp. strain TK-105 and *Cycloclasticus* sp. strain TK-8 with Macondo crude oil.

Hydrocarbon group	Avg. μg g^{-1} oil (±SE) of compound biodegraded by:[a]		
	Strain TK-46(2)	Strain TK-105	Strain TK-8
Aliphatics:			
nC_{12}	1135 ± 699		
nC_{13}	1358 ± 873		
nC_{14}	1193 ± 637		949 ± 496
nC_{15}	436 ± 309		532 ± 244
Aromatics:			
Naphthalene	3349 ± 845	3325 ± 990	6847 ± 20
1-methylnaphthalene	6530 ± 302	1367 ± 999	11721 ± 119
2-methylnaphthalene	9833 ± 302	7915 ± 848	16629 ± 100
1-ethylnaphthalene			603 ± 19
2-ethylnaphthalene	857 ± 19		1769 ± 29
1,2-dimethylnaphthalene	553 ± 184		1699 ± 36
1,5-dimethylnaphthalene	887 ± 108		2171 ± 26
1,6-dimethylnaphthalene	3945 ± 98		7598 ± 117
(1,4+2,3)-dimethylnaphthalene	1990 ± 122		4689 ± 49
(1,3+1,7)-dimethylnaphthalene	6888 ± 308		16111 ± 147
(2,6+2,7)-dimethylnaphthalene	5720 ± 271		12535 ± 127
phenanthrene			6445 ± 136
1-methylphenanthrene			2281 ± 214
2-methylphenanthrene			3037 ± 214
3-methylphenanthrene			3228 ± 215
9-methylphenanthrene			4175 ± 206
(1,3+2,10+3,9+3,10)-dimethylphenanthrenes			2583 ± 417
dibenzothiophene			617 ± 12

[a] Values are the average (Avg) from triplicate measurements and expressed as μg of compound biodegraded g^{-1} oil after 20 days relative to uninoculated controls. SE, standard error. Note: significant degradation of nC_{18} (not included here) was identified when analysed as a ratio of nC_{18}:phytane (see Fig. 1).

Table 2. Monosaccharide composition of the EPS produced by *Alteromonas* sp. strain TK-46(2).

Component [a]	Mean mol% composition [b]
Rha	26.3 ± 2.7
Fuc	trace
Gal	47.8 ± 2.7
GalN [c]	ND
Glc	7.7 ± 1.9
GlcN [c]	1.3 ± 0.3
Man	3.5 ± 0.5
Xyl	ND
GalA	6.3 ± 1.2
GlcA	7.1 ± 0.5
Total (%): [d]	39.2 ± 7.4

[a] Standard abbreviations used are: Rha (rhamnose), Fuc (fucose), Gal (galactose), GalN (galactosamine), Glc (glucose), GlcN (glucosamine), Man (mannose), Xyl (xylose), GalA (galacturonic acid) and GlcA (glucuronic acid).
[b] Values are the mean of triplicate samples ± standard deviation; ND, not detected.
[c] N-Acetylgalactosamine and N-acetylglucosamine are de-N-acetylated during the acid hydrolysis and are detected as galactosamine and glucosamine.
[d] Total % values are expressed as the mean percentage of total dry weight of the polymer from triplicate determinations.

Table 3. Amino acid composition of the EPS produced by *Alteromonas* sp. strain TK-46(2).

Component	Mean mol% composition[a]
Aspartic acid	13.0 ± 0.6
Threonine	6.4 ± 0.2
Serine	6.7 ± 0.3
Glutamic acid	10.6 ± 0.4
Proline	5.2 ± 0.2
Glycine	15.7 ± 0.7
Alanine	12.4 ± 0.6
Cystine	ND
Valine	4.9 ± 0.1
Methionine	0.9 ± 0.0
Isoleucine	2.7 ± 0.1
Leucine	4.9 ± 0.2
Tyrosine	0.8 ± 0.0
Phenylalanine	3.9 ± 0.2
Histidine	8.8 ± 0.4
Lysine	1.9 ± 0.1
Arginine	1.2 ± 0.1
Total (%)[b]	6.4 ± 0.3

[a] Values are the mean of triplicate samples ± standard deviation; ND, not detected.
[b] Total % values are expressed as the mean percentage of total dry weight of the polymer from triplicate determinations.
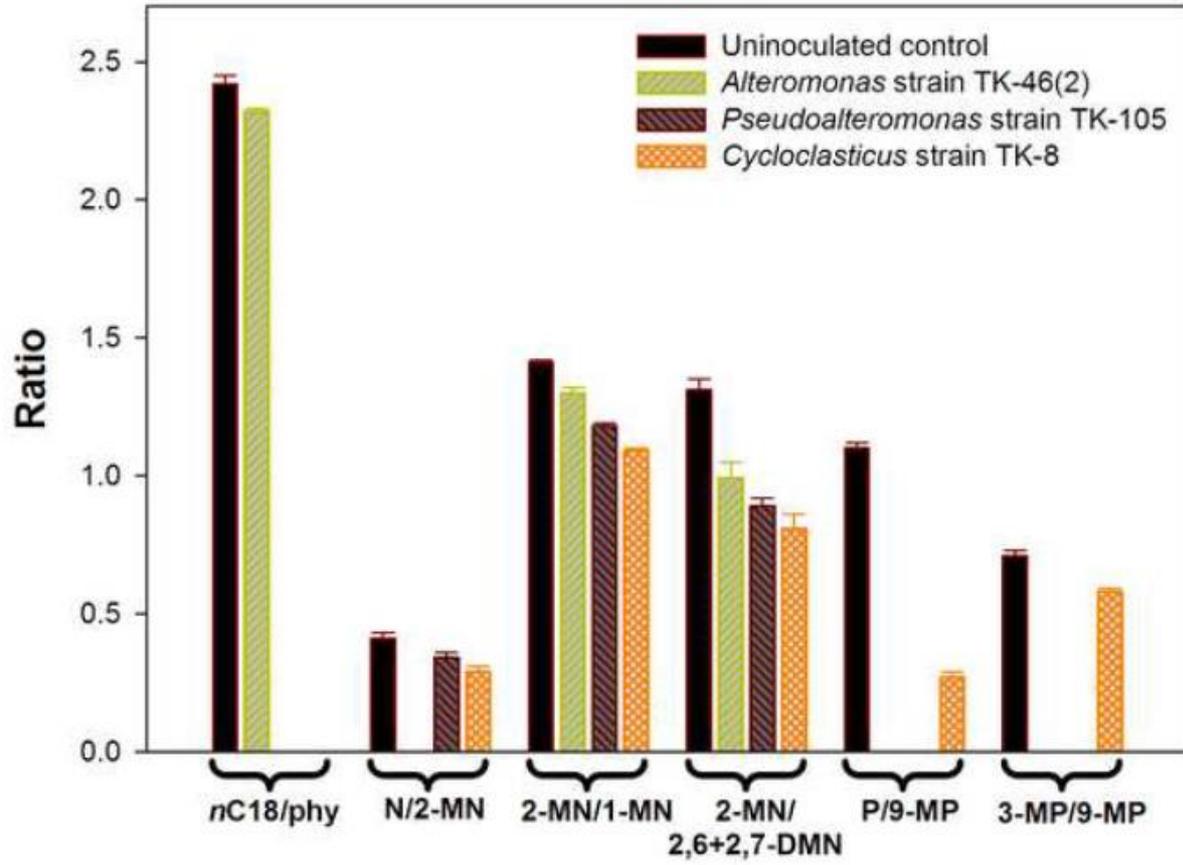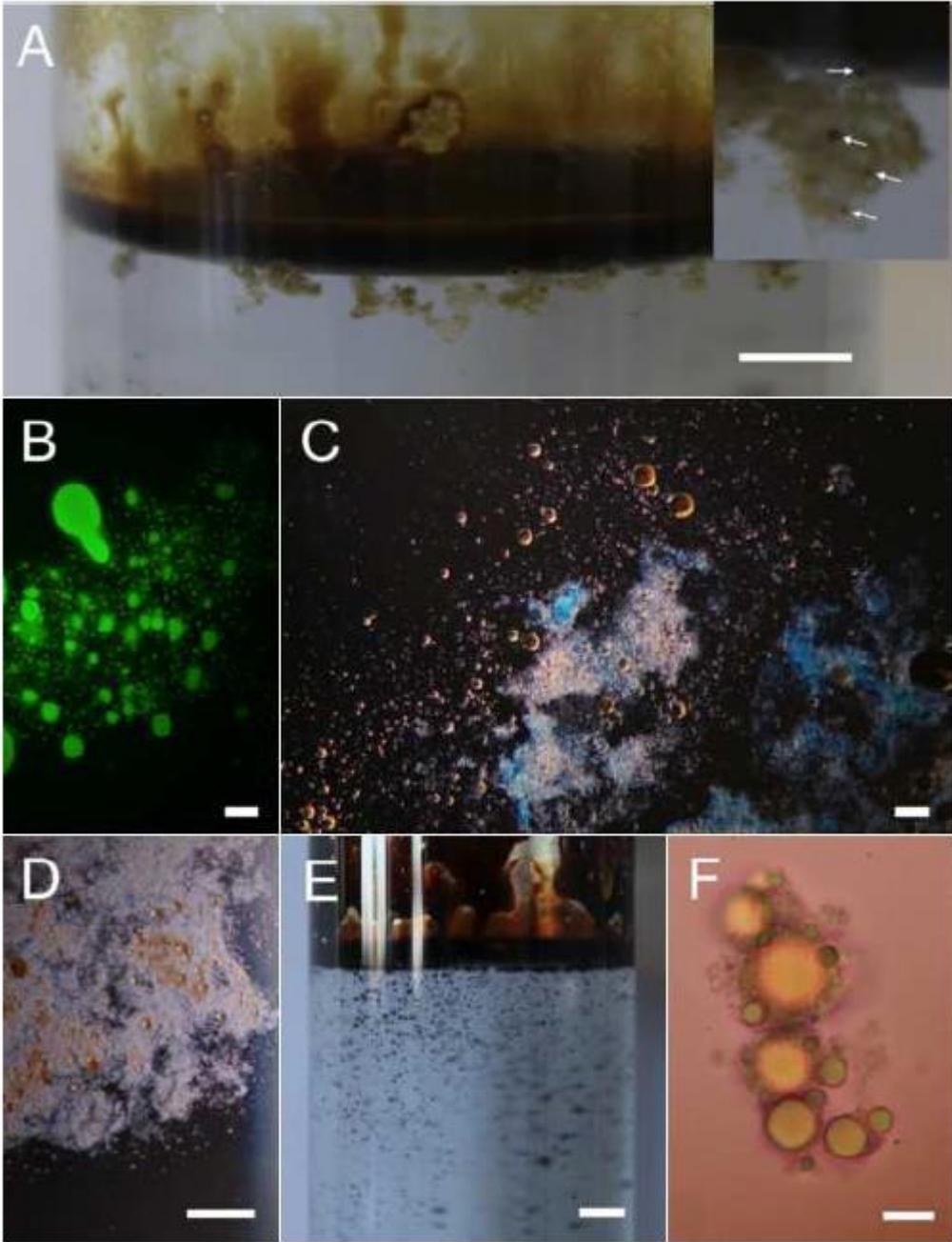
Figure 1

Figure 2

Figure 3