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

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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 enriched in sea surface oil slicks during the spill – contributed to the formation of MOS and/or dispersion of the 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

78 the ocean floor (Hollander et al. 2012; Joye et al. 2014). MOS sedimentation has also been
79 suggested to have originated from the deepwater plume (Valentine et al., 2014). Significant
80 hydrocarbon deposition to the seafloor was observed within 20 km of the spill site (Brooks et al.,
81 2015; Romero et al., 2015; Romero et al., 2017; Stout et al., 2017) and including to the northern
82 Gulf of Mexico (White et al., 2012; Montagna et al., 2013; Valentine et al., 2014; Chanton et al.,
83 2015) as a product of MOS formation (Brooks et al. 2015). Estimates for the amount of weathered
84 oil residues that were transported to the seafloor is comparable between studies, from 1.8-14%
85 (Valentine et al. 2014) to 0.5-9% (Chanton et al. 2015), though still uncertain. Now, just over seven
86 years on, the full environmental impact of this MOS-mediated oil sedimentation remains
87 unresolved.

88 Although conjecture still surrounds what triggered MOS formation during the DwH spill,
89 the prevailing evidence indicates that it was directly associated with the massive influx of crude oil
90 into the Gulf of Mexico. In roller-bottle experiments performed under conditions simulating the
91 Gulf spill by adding weathered oil collected from the sea surface near the Macondo wellhead,
92 Ziervogel et al. (2012) elegantly demonstrated the importance of the oil in MOS formation, and that
93 these amorphous aggregations could act as hotspots of microbial oil-degrading activity that
94 significantly influenced carbon flux in surface oil slicks at DwH. Passow et al. (2012) provided
95 further insight on the complexity of biological interactions that contributed to the formation of
96 MOS, and Bælum et al. (2012) described the formation of flocs (synonymous with MOS) in
97 incubations with seawater and oil from DwH, and showed *Colwellia* was a dominant member of the
98 flocs formed. These studies in the Gulf of Mexico and elsewhere have explored the genesis of MOS
99 formation, revealing the involvement of bacteria, oil, dispersants, mucilaginous polymers (e.g. TEP,
100 EPS) and possibly also eukaryotic phytoplankton (Gutierrez et al. 2013a; Arnosti et al. 2016; Duran
101 Suja et al. 2017; Passow et al., 2012; Fu et al. 2014; Passow, 2016). Whether any one of these
102 biological contributors plays a protagonist role in triggering MOS formation remains

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

105 Marine snow particles are held together by carbohydrate-based polymers, such as
106 transparent extracellular particles (TEP) and/or extracellular polymeric substances (EPS), that can
107 be produced in large quantities by phytoplankton and bacteria. Certain groups of bacteria in the
108 ocean are recognized for producing significant quantities of EPS that contribute to the total
109 dissolved organic matter (DOM) pool in the ocean (Azam, 1998). A large fraction of this bacterial-
110 derived EPS consists of glycoprotein (Long and Azam, 1996; Verdugo et al. 2004), which
111 coincidentally reflects the chemical composition found in MOS that formed in surface and plume
112 waters at DwH (Bælum et al. 2012; Passow et al. 2012; Ziervogel et al. 2012). It can be
113 hypothesized that indigenous groups of EPS-producing bacteria that became enriched during the
114 DwH spill had contributed, via an as yet unknown mechanism(s), to the formation of significant
115 quantities of MOS observed during the spill. Since no methods currently exist that can match any
116 type of EPS in an environmental sample to its biological source, one method to ascertain whether
117 EPS produced by a bacterial group enriched during the DwH spill may had contributed to the
118 formation of MOS is to study them in pure culture. Using roller-bottle incubations under conditions
119 simulating oil-contaminated sea surface water, we previously showed that EPS produced by
120 *Halomonas* sp. TGOS-10 – a hydrocarbon-degrading bacterium that had become enriched in sea
121 surface oil slicks during the DwH spill – could trigger the formation of MOS in the presence of
122 crude oil (Gutierrez et al. 2013a). Other studies also described MOS formation in similar
123 laboratory-based incubations with seawater (Bælum et al. 2012; Passow et al. 2012; Ziervogel et al.
124 2012), but the role of EPS in this respect was not investigated. In this study, we test the hypothesis
125 that other hydrocarbon-degrading bacteria that became enriched in surface oil slicks during the spill
126 were involved in inducing the formation of MOS, as well as participating in the emulsification and
127 degradation of the Macondo oil. MOS formation was evaluated in oil-amended roller bottle
128 incubations in the laboratory over a period of 14 days using constant gentle turbulence to simulate

129 conditions near the sea surface. We also determined the range of hydrocarbons in Macondo oil that
130 these strains are capable of degrading, and we isolated the EPS produced by one of the strains in
131 order to analyse its chemical characteristics and infer on its role in MOS formation and
132 emulsification of the oil.
133

134 **Materials and Methods**

135 *Microorganisms used in this study*

136 *Alteromonas* sp. strain TK-46(2) was originally isolated from a sea surface oil slick sample
137 collected during a research cruise on RV *Pelican* on May 5th of 2010, ca. 0.86 miles from the site of
138 the DwH blowout (28° 44.175' N, 88° 22.335' W). *Pseudoalteromonas* sp. strain TK-105 had been
139 isolated from a deepwater plume sample collected at 1170 m depth (28° 41.686' N, 88° 26.081' W)
140 during a subsequent cruise on the RV *Walton Smith* on 31st May 2010. The strains were selected for
141 use in this study based on their ability to produce EPS, to degrade hydrocarbons, and because they
142 were found enriched (based on 16S rRNA gene sequence identity) in sea surface oil slick samples
143 collected during the DwH oil spill (Gutierrez et al. 2013a,b); the TK-105 strain *Cycloclasticus* sp.
144 strain TK-8 was isolated from the sea surface oil slick and included in this study because, whilst it
145 too was found heavily enriched in sea surface oil slicks and the deepwater plume (Gutierrez et al.
146 2013b), this strain does not produce EPS and therefore served as a useful reference organism to
147 compare with the EPS-producing strains TK-46(2) and TK-105. In addition, it had not previously
148 been determined, by empirical investigation, the range of hydrocarbons in the Macondo crude oil
149 that each of these strains is able to degrade, so was assessed here.
150

151 *Hydrocarbon analysis*

152 To determine the hydrocarbon species in Macondo crude oil that strains TK-8, TK-46(2) and TK-
153 105 can degrade, a synthetic seawater medium, ONR7a (Dyksterhouse et al. 1995) was used and
154 amended with surrogate Macondo crude oil (from the Marlin platform) as the sole carbon and

155 energy source. For this, 250-ml pre-autoclaved glass Schott bottles were prepared containing 45 ml
156 ONR7a, Macondo oil to ca. 100 mg/L final concentration, and inoculated with 5 ml of washed cells.
157 For preparation of inocula, the strains were grown in ONR7a amended with Na-pyruvate (for TK-
158 46(2) and TK-105) or phenanthrene (for TK-8); the cell biomass was washed three times,
159 resuspended in sterile ONR7a to 5 ml and used for inoculation. Additional incubations were set up
160 in the same way with the exception that 85% phosphoric acid (3% final concentration) was added,
161 or the bottles were not inoculated; these controls served to analyse for any loss of hydrocarbons due
162 to abiotic factors. All incubations were carried out in triplicate and incubated in parallel in the dark
163 with gentle shaking (100 rpm) and at 21°C, which is a temperature similar to that at the sea surface
164 in the Gulf of Mexico during the time of the DwH oil spill. At the termination of the experiment
165 (day 20), all the bottles were extracted for total petroleum hydrocarbons (TPH) and subsequent
166 analysis for individual hydrocarbon constituents by gas chromatography/mass spectrometry (GC-
167 MS), as detailed below.

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

173 A known aliquot corresponding to ca. 30mg was taken from the remaining sample and
174 transferred to a 10ml vial. An aliquot of the reference oil was weighed directly into a vial and
175 diluted with ca. 0.3ml DCM. Squalane and 1,1'-binaphthyl were added as standards at ca. 0.5% and
176 0.05% by weight of the oil, respectively. A procedural blank including the standards was also
177 prepared. One sample was analysed in triplicate and the reference oil was analysed in duplicate.

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

181 to the top of the column. The total petroleum hydrocarbon (TPH) fraction was eluted with 50ml
182 petroleum ether followed by 70ml petroleum ether/DCM (2:5). Solvent was reduced to 3ml using a
183 Heidolph rotary evaporator and an aliquot was removed for gas chromatographic analysis.
184 The TPH fractions were analysed on a Hewlett Packard 5890 GC fitted with a split/splitless
185 injector (300°C), a flame ionisation detector (FID) (310°C) and an HP-5 capillary column (J&W,
186 30m x 0.25mm i.d. x 0.25µm film thickness). Samples were injected using a Hewlett Packard 6890
187 automatic injector. The oven programme was 50°C (2 min) – 5°C/min – 300°C (20 min) giving a
188 total run time of 74 min. Chromatographic data were acquired and processed using an Atlas 8.3
189 Chromatographic Data System (Thermo Scientific). Peak areas for individual C_8 to C_{35} *n*-alkanes,
190 the isoprenoids pristane and phytane, and for the added standard squalane were obtained. The total
191 hydrocarbon content was calculated using the manually integrated area under the whole
192 chromatogram, excluding the solvent peak. The corresponding total area for the procedural blank
193 (which also contained the added standards) was then subtracted from the total area obtained for the
194 samples and reference oil. Analyte concentrations were measured using the areas of the added
195 standards, assuming a response factor of one, and are thus semi-quantitative. Full quantitation
196 (using a range of standards and individual analyte response factors) was not carried out since the
197 purpose of the experiments was a comparison between different treatments, including controls,
198 using the same analytical protocols. The aromatic hydrocarbons in the TPH fractions were analysed
199 by GC-MS on an Agilent 7890A GC fitted with a split/split less injector (at 280°C) linked to an
200 Agilent 5975C MSD, with data acquisition and processing by Agilent Chemstation software.
201 Selected samples were analysed in full scan mode (50-600 amu sec^{-1}) but all samples were analysed
202 in selected ion monitoring (SIM) mode using the analyte aromatic hydrocarbon molecular ions or
203 major fragment ions. An aliquot (1 µl) of the TPH fraction diluted in hexane/dichloromethane was
204 injected in split/splitless mode using an Agilent 7683B autosampler and the split opened after 1
205 minute. Separation was performed on an Agilent fused silica capillary column (30 m x 0.25 mm i.d)
206 coated with 0.25 µm 5% phenylmethylpolysiloxane (HP-5) phase. The GC was temperature

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

212 Ratios of *n*-alkanes to acyclic isoprenoid hydrocarbons (nC_{17}/pristane and nC_{18}/phytane)
213 were used as convenient indicators of biological degradation, due to the recalcitrance imparted by
214 the branched structure of the isoprenoid biomarkers (Sauer and Boehm, 1991; Papazova and
215 Pavlova, 1999; Dawson et al., 2013). Similarly for aromatic hydrocarbon analysis, this was done for
216 7 ratios indicative of biodegradation (naphthalene/2-methylnaphthalene; 2-methylnaphthalene/1-
217 methylnaphthalene; 2-ethylnaphthalene/2,6+2,7-dimethylnaphthalene; 2-
218 methylnaphthalene/2,6+2,7-dimethylnaphthalene; phenanthrene/9-methylphenanthrene; 3+2-
219 methylphenanthrene/9+1-methylphenanthrene; 3-methylphenanthrene/9-methylphenanthrene).
220 Concentrations of aliphatic and aromatic hydrocarbon species/groups that were biodegraded after
221 20 days were also calculated by subtracting the respective hydrocarbon concentrations measured in
222 the acidified controls from those of the non-acidified incubations. A Student's *t*-test was performed
223 to test for significant differences ($P < 0.05$) in the degradation of the hydrocarbons by each strain
224 analysed against their respective uninoculated/acid-inhibited controls.
225
226 *Roller-bottle incubation experiments*
227 The potential of strains TK-46(2), TK-105 and TK-8 in promoting the formation of MOS and/or
228 emulsification of oil was investigated using a roller-bottle design, as described previously
229 (Gutierrez et al. 2013a). This type of experimental setup maintains the system in a constant gentle
230 turbulence, thereby reducing the potential of particles to settle on the container walls (Jackson,
231 1994) and simulates conditions near the sea surface. Autoclaved synthetic seawater, ONR7a, was
232 utilized in these experiments to directly associate the formation of any MOS and/or emulsions to the

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

235 For each strain, three roller-bottle experiments were run in duplicate. The first involved the
236 use of inactive/non-respiring cells (Na-azide treated) to examine the potential of the cell surface
237 alone to promote the formation of MOS or the emulsification of the oil – hereafter referred to as the
238 azide-inhibited incubations. The second employed using cell-free fractions to determine if any
239 extracellularly-released EPS from these strains promoted the same effect – hereafter referred to as
240 the cell-free fraction incubations. The third experiment used live cells to evaluate whether cell
241 activity, such as degradation of the oil by the strains, might promote MOS and/or emulsion
242 formation – hereafter referred to as the live-cell incubations. The inoculum for each of these
243 experiments was prepared by growing up a large batch of cells in ONR7a amended with filter-
244 sterilized (0.2 μm) Macondo oil (0.5% v/v final concentration), glucose (0.05% w/v final
245 concentration), and a trace elements and vitamin mixture (Dyksterhouse et al. 1995). The cell
246 biomass was recovered by centrifugation (8,000 x g; 20 min) and the supernatant fraction set aside.
247 The cells were washed twice with autoclaved ONR7a, re-suspended to a final optical density (600
248 nm) of 0.06, and supplemented with Na-azide (0.01% w/v final concentration) to render the cells
249 inactive for use in the azide-inhibited incubations. For preparation of the cell-free fraction
250 incubations, the above supernatant fractions for each respective strain were filtered (0.2 μm) in
251 order to remove all residual cells and then supplemented with Na-azide as above. Washed 'live'
252 cells (i.e. in the absence of Na-azide treatment) were used (0.06 final OD_{600}) for the live-cell
253 incubations. Each of the three incubations was conducted in duplicate using 250-ml Pyrex© glass
254 bottles (27 x 37 mm) which were filled with 200 ml of the respective inoculum fraction, and to each
255 1 ml of sterile oil was added to a final oil slick content of 0.5% (v/v). Control incubations were run
256 in parallel using uninoculated autoclaved ONR7a in the presence or absence of oil. Inoculated
257 controls were also included, but without the addition of oil. The bottles were incubated at 21°C in
258 the dark on a roller table at 3.5 rpm for 14 days. The bottles were periodically placed upright to

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

262 At the end of these 14-day roller bottle incubations, MOS particles that formed were
263 carefully withdrawn using glass Pasteur pipettes and transferred to 1.5-ml microtubes for staining
264 with the cationic copper phthalocyanine dye alcian blue (AB) at pH 2.5 (Alldredge et al. 1993), or
265 with the amino acid-specific dye coomassie brilliant blue G (CBBG) at pH 7.4 (Long and Azam,
266 1996). AB is used for staining acidic sugars of EPS, whereas CBBG is used for staining the
267 proteinaceous component of EPS. Following staining, the MOS particles were washed by
268 transferring them through several droplets of sterile water prior to their examination under the light
269 microscope.

270

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

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

280

281 *Chemical analysis of the TK-46(2) EPS*

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

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

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

299 For molecular weight and polydispersity determination of polymer TK-46(2), size-exclusion
300 chromatography coupled to multi-angle laser light scattering (SEC-MALLS) was used. For this, the
301 polymer was dissolved in distilled water at ~0.3% (wt/vol) and then analysed by size exclusion
302 chromatography at ambient temperature on a PL Aquagel guard column (Polymer Labs, Amherst,
303 U.S.A.) which was linked in series with PL Aquagel-OH 60, PL Aquagel-OH 50 and PL Aquagel-
304 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
305 online firstly by a DAWN EOS light scattering detector (Wyatt Technology, Santa Barbara, U.S.A.)
306 and by a rEX differential refractometer (Wyatt Technology, Santa Barbara, U.S.A.). The refractive
307 index increment, *dn/dc* was taken to be that of a typical polysaccharide (0.150 ml/g) (Harding et al.,
308 1991; Theisen et al., 2000). Samples were run in triplicate.

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

311 spectra were acquired at 60°C using a Bruker AVIII 400 MHz spectrometer. Temperature
312 regulation utilized a BVT3200 temperature control unit. One-dimensional spectra were acquired
313 using the Bruker pulse program 'zgesp' featuring a water-suppression sequence. The number of
314 scans was set at 256, the acquisition time was ca. 1 second and a line-broadening factor of 1Hz was
315 imposed on the data prior to processing. TOCSY spectra were acquired using the Bruker pulse
316 program 'dipsi2esgpph' featuring a water-suppression sequence. TD(1) was set at 256W and the
317 data was truncated in f_2 with TD_{eff} set to 800W, TD(2) being set to 2048W. The mixing time was
318 set to 120ms. COSY spectra were acquired using the Bruker pulse program 'cosydfgpph19'
319 featuring a water-suppression sequence. TD(1) was set at 256W and the data was truncated in f_2
320 with TD_{eff} set to 800W, TD(2) being set to 2048W.
321
322 *Emulsification assays*
323 To evaluate the potential of strains TK-46(2), TK-105 and TK-8 to produce surface-active agents
324 such as bio-surfactants or bio-emulsifiers, they were incubated in ONR7a or ZM/100 marine
325 medium supplemented with Macondo oil (0.05% v/v) or glucose (0 to 0.5% w/v). After 3-4 days
326 incubation (120 rpm; 21°C), emulsification assays and surface tension measurements were
327 performed on cell-free fractions of the cultures, as previously described (Gutierrez et al. 2007).
328 Briefly, to assay for emulsification activity, the samples were mixed with an equal volume of the oil
329 (Macondo oil or *n*-hexadecane) in acid-washed (0.1 N HCl) screw-cap glass tubes (100 x 13 mm),
330 manually shaken (15 s) and vortexed (15 s) to homogeneity, left to stand for 10 min, shaken as
331 before, and the height of the emulsion layer – expressed as Emulsification Index, EI_{24} – measured
332 after allowing the mixture to stand for 24 h at 21°C. For tensiometry, a Kibron Ez-Pi Plus
333 tensiometer was used with a Kibron Dyne Probe for static du Noüy measurements.
334

335 Results

336 *Degradation of Macondo crude oil by the strains*

337 Hydrocarbon degradation tests showed that the *Alteromonas*, *Pseudoalteromonas* and
338 *Cycloclasticus* strains degraded distinct substrate classes in the Macondo oil. For this, the total
339 volume of each of the cultures for each strain were extracted and analysed for total petroleum
340 hydrocarbons (TPH) and their hydrocarbon composition, as described below. Compared with
341 uninoculated/acid-inhibited controls, the concentrations of some hydrocarbon species were found to
342 have significantly decreased ($P < 0.05$) after 20 days in the 'live' (no acid treated) incubations, and
343 could thus be attributed to biodegradation by the strains. Table 1 shows these hydrocarbons that
344 were significantly biodegraded for each of the three strains. Of a total of 28 *n*-alkanes analysed
345 (nC_8–nC_{35}), five were significantly degraded (i.e. nC_{12}–nC_{15}) by strain TK-46(2), and another two
346 (nC_{14}, nC_{15}) by strain TK-8 (Table 1). All other *n*-alkanes were either not biodegraded or their loss
347 was accounted for by abiotic degradation respective to the controls. From these same experiments,
348 ratios of *n*-alkanes to acyclic isoprenoid biomarkers, and similarly ratios for aromatic hydrocarbons,
349 were used as indicators of biological degradation (Figure 1). For strain TK-46(2), reductions in
350 nC_{18}/phy, 2-methylnaphthalene/1-methylnaphthalene and 2-methylnaphthalene/2,6+2,7-
351 dimethylnaphthalene were measured. For strain TK-105, reductions in naphthalene/2-
352 methylnaphthalene, 2-methylnaphthalene/1-methylnaphthalene and 2-methylnaphthalene/2,6+2,7-
353 dimethylnaphthalene were measured. For strain TK-8, reductions in naphthalene/2-
354 methylnaphthalene, 2-methylnaphthalene/1-methylnaphthalene, 2-methylnaphthalene/2,6+2,7-
355 dimethylnaphthalene, phenanthrene/9-methylphenanthrene and 3-methylphenanthrene/9-
356 methylphenanthrene were measured. No significant reductions for nC_{18}/phytane, nC_{17}/pristane, 2-
357 ethylnaphthalene/2,6+2,7-dimethylnaphthalene and 3+2-methylphenanthrene/9+1-
358 methylphenanthrene were measured. These results, as expected, show that strain TK-8 excelled in
359 degrading the aromatic hydrocarbon fraction of the oil compared to the other two strains. Taken
360 collectively, the strains exhibited a capacity to degrade a wide range of hydrocarbon species, with
361 strain TK-8 specialising in the degradation of aromatic hydrocarbons. Higher-molecular-weight
362 aromatic compounds, such as substituted/non-substituted dibenzothiophenes, monoaromatic

363 steroids and triaromatic steranes (measured from their respective ion chromatogram peak areas
364 against that of the added 1,1'-binaphthyl, using a response factor of one), did not appear to be
365 degraded by any of the three strains, at least under the experimental conditions employed.
366
367 *MOS formation and Macondo oil emulsification potential by the strains*
368 In live-cell roller bottle incubations with *Alteromonas* strain TK-46(2) incubated with synthetic
369 seawater medium ONR7a (Dyksterhouse et al. 1995) amended with filter-sterilized Macondo crude
370 oil, MOS formation was observed after 5 days with the abundance of MOS aggregates increasing
371 until day 12 (Fig. 2A). Similar observations were recorded for this strain in the azide-inhibited
372 incubations, with the exception that MOS formation did not occur until day 7. For both the live-cell
373 and azide-inhibited incubations, the diameter of the MOS particles formed ranged from 2 to 5 mm.
374 They displayed a 'fluffy' off-white appearance and none of the particles settled to the bottom when
375 the bottles were held upright – all the particles floated and localised just under the partially
376 emulsified oil slick. Upon close inspection, they were found to contain small associated oil droplets
377 (inset to Fig. 2A). When submerged within the bottles after applying a gentle mixing, the MOS
378 particles floated slowly back up and settled just under the oil slick, and at no time over the duration
379 of these 14-day incubations did they settle to the bottom of the bottles. When stained with Acridine
380 Orange (AO) and viewed under the epifluorescence microscope, the MOS particles from both
381 incubations treated with or without azide were found loaded with attached cells and oil droplets
382 (Fig. 2B). When viewed under the light microscope with the aid of dark field illumination, the
383 particles appeared as an amorphous 'cotton wool-like' material that partially stained with the
384 polysaccharide-specific dye Alcian Blue (AB), and were seen floating in a 'sea' of emulsified oil
385 droplets (dark brown spheres; average size range <1 to 5 µm i.d.), of which many appeared
386 embedded within the MOS particles (Fig. 2C). The MOS particles, however, did not stain with the
387 protein-specific dye Coomassie Brilliant Blue G (CBBG) (Fig. 2D). Conversely, no formation of

388 MOS or emulsification of the oil was observed in roller bottles incubated with cell-free fractions of
389 strain TK-46(2).
390 In roller bottle incubations containing live cells, azide-inhibited cells, or cell-free fractions
391 of strain *Cycloclasticus* strain TK-8 or *Pseudoalteromonas* strain TK-105, no MOS was observed to
392 have formed at any point during these 14-day incubations. However, in incubations with TK-105,
393 the oil had shown signs of partial emulsification by day 3, and became progressively more
394 emulsified over the next few days until the termination of these experiments. Increasing
395 emulsification was indicated by the presence of small oil droplets that appeared submerged just
396 below the oil slick when the bottles were held upright and gently agitated (Fig. 2E). In none of these
397 incubations had the oil become completely emulsified since a clear slick of non-emulsified oil
398 remained intact at the termination of these experiments. Light microscopic examination of the
399 emulsified oil droplets located under the oil slick did not reveal the presence of TK-105 cells
400 associated with the oil (not shown). Conversely, oil droplets withdrawn from live-cell incubations
401 with TK-8 and viewed under the light microscope revealed they were coated with a dense
402 population of the cells (Fig. 2F). Oil droplets in these incubations with TK-8 were sparse, and
403 essentially the oil slick layer did not appear emulsified in any of the roller bottle incubations that
404 contained live cells, azide-inhibited cells or cell-free fractions of this strain. It is worthy to note that
405 the cell-free fraction for this strain, prepared from growth of TK-8 in ONR7a amended with
406 Macondo oil, exhibited an orange appearance, which is indicative of the extracellular accumulation
407 of an oxidized intermediate(s) from the metabolism of aromatic hydrocarbons (e.g. PAHs) in the
408 Macondo oil (Cuskey and Olsen, 1988).
409 In uninoculated control incubations with ONR7a amended with oil only, or ONR7a
410 inoculated with either of the three strains in the absence of oil, no signs of MOS formation or
411 emulsification of the oil were observed at any point over the course of these 14-day experiments
412 (results not shown).
413

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

415 A monosaccharide analysis of the EPS produced by *Alteromonas* sp. strain TK-46(2) showed that it
416 contained a carbohydrate content of 39.2 ± 7.4% of the total weight of dried polymer (Table 2). The
417 polymer was composed of hexoses (rhamnose, fucose, galactose, glucose, mannose), amino sugars
418 (glucosamine), uronic acids (galacturonic and glucuronic acid), and the pentose xylose. Rhamnose
419 (26.3% ± 2.7%) and galactose (47.8% ± 2.7%) were the most abundant. All other monosaccharides
420 were each present at less than 10% and together they contributed about 25.9% ± 0.9% to the total
421 carbohydrate content, with trace quantities of fucose detected. The total uronic acid content of the
422 TK-46(2) EPS was 13.4%, as contributed by galacturonic and glucuronic acids.

423 The total amino acid content of the TK-46(2) EPS was 6.4% ± 0.3% (Table 3) of the total
424 weight of dried polymer. Amino acid analysis of hydrolyzed samples identified the presence of four
425 major amino acids – aspartic acid, glutamic acid, glycine, and alanine – that in total contributed
426 51.7% ± 0.6% to the total amino acid content. The percent contribution of hydrophobic nonpolar
427 amino acids to the total amino acid content was 50.6%, whereas that of polar amino acids was
428 49.4%. Lipid analysis did not reveal any fatty acids.

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

440 smaller molecular-weight species. This behaviour can result from the high content of anionic
441 moieties, such as uronic acids, which are typically enriched in marine bacterial EPS (Decho and
442 Gutierrez, 2017).

443 Figure 3A displays the expansion of the 1D ^{1}H NMR spectrum of the TK-46(2) EPS
444 showing strong peaks from the glycan component predominating and the typical appearance of a
445 complex carbohydrate spectrum. There is a downfield group of at least 6 fairly well resolved
446 anomeric signals between 4.7 and 5.5 ppm, characteristic of the α configuration at the anomeric
447 centre. An envelope of overlapping signals between 3.3 and 4.3 ppm originates from the ring and
448 methylene protons of sugars. A strong group of signals at about 1.3 ppm may arise from protons at
449 rhamnose C6 (see Table 2); a peak near 2.05 ppm may include the N-acetyl methyl signal, from the
450 small proportion of glucosamine in the sample (see Table 2). A sharp peak at 1.5 ppm may arise
451 from residual solvent, and the acetone internal standard signal is seen at 2.22 ppm. The small
452 proportion of amino acids in the sample (Table 3) is also reflected in the ^{1}H NMR spectrum, though
453 signals from methyl groups of alanine and threonine coincide with the rhamnose methyl in the
454 envelope of peaks near 1.3 ppm. The TOCSY spectrum displayed in Figure 3B shows a group of
455 cross-peaks linking the anomeric and ring protons, and a further group of cross-peaks linking the
456 signals assigned as H6 of rhamnose with the ring proton region. An unidentified component gives
457 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
458 the sugars listed in Table 2; this group of signals may possibly be attributed to the presence of
459 proline. The six major and several other minor signals in the anomeric region of the spectrum
460 indicate a complex structure for the polysaccharide, with some degree of heterogeneity. This
461 structural spectrum is comparable with exopolysaccharides from other *Alteromonas* strains (Le
462 Costauëc et al. 2012).
463
464 *Surface-active qualities of the TK-46(2) EPS*

465 Cell-free fractions of the strains TK-46(2) and TK-105, during growth in ONR7a or ZM/100
466 medium amended with glucose, resulted in partial emulsification of Macondo oil or *n*-hexadecane
467 in emulsification assays. No emulsification occurred with cell-free extracts of strain TK-8. The
468 lyophilized TK-46(2) polymer was dissolved in seawater at a concentrations of 0.2% (w/v) and the
469 solution was used to perform the emulsification assay and measure for any reduction to the surface
470 tension of seawater. The polymer was found to strongly emulsify crude oil and the hydrocarbon oil
471 *n*-hexadecane, resulting in EI_{24} values of 100%, indicating that the oil layer had been completely
472 emulsified in these assays. Neither the lyophilized polymer nor cell-free fractions of the three
473 strains resulted in a marked reduction to the surface tension of seawater to below 62.5 mN/m at
474 21°C.
475

476 **Discussion**

477 Hydrocarbon analysis revealed that all three strains (*Alteromonas* TK-46(2), *Pseudoalteromonas*
478 TK-105 and *Cycloclasticus* TK-8) were able to degrade various aliphatic and aromatic
479 hydrocarbons of the Macondo oil. As expected, strain TK-8 degraded a range of aromatic
480 hydrocarbons, which is typical for the genus *Cycloclasticus* (Head et al., 2006; Yakimov et al.,
481 2007). Notably, however, we found this strain also able to degrade aliphatics, specifically nC_{14} and
482 nC_{15}, representing the first direct evidence for *Cyclocasticus* to have contributed to the degradation
483 of the saturated fraction of hydrocarbons in the Macondo oil; this genus had previously been
484 recognised to specialise in the degradation of PAHs only. Supporting this new ecophysiological
485 role, a recent study analysing reconstructed genomes of several dominant hydrocarbon-degrading
486 taxa that were enriched during the DwH spill showed that *Cycloclasticus* enriched on PAH
487 substrates in stable isotope probing experiments encoded a near-complete *n*-alkane degradation
488 pathway as well (Dombrowski et al. 2016). Another recent study also showed members of
489 *Cycloclasticus*, in this case that were found living in symbiosis with mussels and sponges in deep-
490 sea gas and oil seeps, to be capable of utilising short-chain alkanes (Rubin-Blum et al., 2017).

491 These results from mutually independent investigations indicate that polycyclic aromatic carbon
492 sources do not represent the full substrate spectrum of *Cyclcoclasticus* (Head et al. 2006; Yakimov
493 et al. 2007) in the environment. It should also be noted that preferential degradation of C_6-C_{15} *n*-
494 alkanes before C_{15+} *n*-alkanes is known to occur in subsurface petroleum reservoirs (Larter et al.
495 2012). The ability of strain TK-8 to metabolise substituted polycyclic aromatic compounds together
496 with *n*-alkanes strongly suggests that members of *Cycloclasticus* had contributed not just to the
497 degradation of the aromatic hydrocarbons of the Macondo oil, but also the aliphatic fraction; though
498 likely less significantly for the latter as species of this genus are mainly recognized for their almost
499 exclusive preference for utilizing aromatic hydrocarbons (Head et al., 2006; Yakimov et al., 2007).
500 Taken together, the versatility of strain TK-8 to degrade both aromatic and aliphatic hydrocarbons,
501 its affinity for attaching to oil droplets, and the strong enrichment of *Cycloclasticus* populations
502 during the DwH spill, identifies this genus as a major protagonist in the biodegradation of the
503 Macondo oil.
504 Interestingly, our hydrocarbon analysis did not reveal strains TK-46(2) or TK-105 capable
505 of degrading phenanthrene in the Macondo oil, yet both strains were previously shown to utilise this
506 compound as a sole carbon source (Gutierrez et al. 2013b). Enrichments with complex hydrocarbon
507 mixtures, such as crude oil, can result in competitive inhibition (Stringfellow et al. 1995), and could
508 explain why both strains did not show significant degradation of phenanthrene in the Macondo oil,
509 even though their ability to utilise this compound has been substantiated in pure culture experiments
510 (Gutierrez et al. 2013b). Furthermore, whilst strain TK-105 was not identified to significantly
511 degrade any *n*-alkane species, it is possible that this 'generalist' hydrocarbon degrader may encode
512 the capacity to do so, but this was not possible due to competitive inhibition effects.
513 Species of *Pseudoalteromonas* and *Alteromonas* are important members of the 'generalist'
514 hydrocarbon-degrading community in marine environments, and these organisms were enriched
515 during the DwH spill by at least one order of magnitude compared to their abundance in
516 uncontaminated reference waters (Gutierrez et al., 2013a); they have also been found abundant in

517 natural hydrocarbon seep sites in the Gulf of Mexico (Kleindienst et al. 2016). Considering the
518 confirmed hydrocarbon-degrading ability of *Alteromonas* TK-46(2) and *Pseudoalteromonas* TK-
519 105, these organisms, together with *Cycloclasticus*, had likely contributed significantly to the
520 degradation of the Macondo oil in sea surface oil slicks where they became strongly enriched
521 (Hazen et al., 2010; Gutierrez et al., 2013b; Valentine et al., 2010). It is possible that these
522 *Pseudoalteromonas* and *Alteromonas* strains contributed here in part through their known ability to
523 produce EPS exhibiting surface-active properties (i.e. acting as bioemulsifiers or biosurfactants),
524 which in turn are known to enhance the dispersion and aqueous solubility of hydrocarbons (Bozzi *et*
525 *al.*, 1996; Cambon-Bonavita *et al.*, 2002; Colliec Jouault *et al.*, 2001; Gutierrez *et al.*, 2007;
526 Gutierrez *et al.*, 2008; Mancuso Nichols *et al.*, 2004; Marx *et al.*, 2009; Qin *et al.*, 2007; Raguenes
527 *et al.*, 1996). This enhanced-dissolution effect may have consequently promoted the degradation of
528 hydrocarbons by these and other bacteria like the dominant *Cycloclasticus*. As the emulsification of
529 crude oil is a natural weathering process, the *de novo* synthesis of surface-active EPS by these
530 strains, in particular TK-46(2) and TK-105, may have contributed to the observed emulsification of
531 the crude oil slick in these bottle experiments, albeit at varying levels of emulsification.
532 Whilst not recognised for producing EPS, the strong enrichment of *Cycloclasticus* in sea
533 surface oil slicks during the DwH spill may have been influenced by the surface-active properties of
534 EPS produced by the EPS-producers *Alteromonas*, *Pseudoalteromonas*, and the previously
535 described *Halomonas* sp. strain TGOS-10 (and other halomonads) that was also enriched in sea
536 surface oil slicks (Gutierrez et al., 2013a). Our analysis of the carbohydrate component of the TK-
537 46(2) EPS revealed it to have a relatively high uronic acid content of 13.4%. The carboxylate and
538 methoxycarbonyl groups of these types of acidic sugars can mediate the adsorption of EPS to oil
539 droplets and form stable emulsions (Dea and Madden 1986; Kaplan et al. 1987; Tolstogusov 1991,
540 1994), which may explain the oil emulsions that formed in roller-bottle incubations with live and
541 azide-inhibited cells of strain TK-46(2) and TK-105. The presence also of 6-deoxyhexoses (e.g.
542 rhamnose) and increased substitution by acetyl moieties (NMR peak near 2.05 ppm) on the TK-

543 46(2) EPS can also render these types of polymers with surface-active qualities (Dea and Madden
544 1986; Graber et al. 1988).

545 Conjecture still surrounds the mechanism underlying MOS formation at DwH, as well as
546 during the Ixtoc-I (Boehm and Fiest, 1980; Jernelöv and Linde ́n, 1981; Patton et al., 1981) and
547 *Tsesis* (Johansson et al., 1980) oil spills where its formation has been proposed (Teal and Howarth,
548 1984). Several reports provide evidence to implicate bacterial EPS in its formation (Gutierrez et al.,
549 2013a; Passow *et al.*, 2012; Ziervogel *et al.*, 2012), possibly via the physicochemical interaction
550 between oil droplets and bacterial EPS (Bælum *et al.*, 2012; Passow *et al.*, 2012). Corroborating
551 with the results from these studies, our roller-bottle experiments with *Alteromonas* sp. strain TK-
552 46(2) showed that the EPS it produced played a role in MOS formation, and that the presence of
553 bacterial cells (in our case with strain TK-46(2)) was required also for this process to occur
554 (Gutierrez et al., 2013a; this study) since incubations with EPS alone (i.e. cell-free fractions) did not
555 yield MOS. Further supporting the role for strain TK-46(2) and its EPS in the formation of MOS
556 during the early phase of the DwH spill, we isolated a *Alteromonas* strain (matching strain TK-
557 46(2) with 100% 16S rRNA gene sequence identity) from a MOS particle that formed in roller-
558 bottle incubations with sea surface water collected during the active phase of the DwH spill
559 (unpublished data). Distinct bacterial phylotypes found on MOS particles compared to those in the
560 surrounding seawater have been observed in experiments assessing MOS formation in surface
561 waters of the Gulf of Mexico (Arnosti et al., 2016) and of the northeast Atlantic (Duran Suja et al.,
562 2017). In these studies, MOS particles were found enriched with various different genera of
563 bacteria, including EPS-producers such as *Halomonas*, *Pseudoalteromonas* and *Alteromonas*,
564 corroborating our results that showed MOS particles heavily colonized with cells of strain TK-
565 46(2).

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

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

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

595 Atlantic surface seawater environment (Duran Suja et al., 2017). The 16S rRNA gene sequence of
596 strain TK-8 was a 100% and 98% match to that of *Cycloclasticus* clones Oil-BE-081 and Oil-BE-
597 051, respectively, that were identified enriched in MOS particles reported by Arnosti et al. (2016),
598 thus implicating this organism in playing a pivotal role in the biodegradation of MOS-associated oil
599 during the spill. Supporting this, *Cycloclasticus* was also found in high abundance in freshly-
600 deposited seafloor sediments in the Gulf following the DwH spill (Yang et al. 2016b). The
601 enrichment of oil-degrading bacteria in MOS is important for understanding the fate of crude oil in
602 the event of an oil spill at sea and warrants further investigation.

603 Positive staining of the MOS particles with AB, and not CBBG, provided evidence of their
604 composition as predominantly polysaccharide. This agrees with chemical characterization, using
605 monosaccharide, amino acid and proton NMR analyses, of the TK-46(2) EPS, which will have
606 dominated the composition of the total polymeric matter that comprised the MOS particles. The low
607 protein content (6.4% of total polymer; Table 3) of the EPS produced by this strain is consistent
608 with that of other marine *Alteromonas* species (Rougeaux et al., 1996; Cambon-Bonavita et al.,
609 2002; Raguenes et al., 2003), as also with the chemical composition of marine EPS, which makes
610 up much of the dissolved organic matter (DOM) in the ocean and is largely derived from
611 phytoplankton and bacterial exudates (Gutierrez et al., 2007; Hassler et al., 2011; Mancuso Nichols
612 et al., 2004). Glucose is the most abundant simple sugar in the ocean, with concentrations averaging
613 from 0.001 to 1.0 μmol/L (Benner, 2002; Skoog et al, 2002; Rich et al, 1996, 1997), and as high as
614 187 nM measured in unfiltered water of the Gulf of Mexico (Skoog and Benner, 1997). Hence, a
615 proportion of the DOM, as EPS macromolecules, in surface waters of the Gulf of Mexico prior to
616 the onset of the DwH oil spill may likely have been the product from the metabolism of this
617 endogenous source of glucose by EPS-producing bacteria, like *Alteromonas*, *Pseudoalteromonas*
618 and *Halomonas*, that were present in surface waters of the Gulf of Mexico (Gutierrez et al.,
619 2013a,b; Yang et al., 2016a). A study by Eenennaam et al. (2016) showed that EPS released by
620 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.

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930 **Figure legends**

931

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

940

941 **Figure 2.** Formation of marine oil snow (MOS) and/or emulsions by *Alteromonas* sp. strain TK-
942 46(2), *Pseudoalteromonas* sp. strain TK-105 and *Cycloclasticus* sp. strain TK-8 in roller bottle
943 incubations. MOS formed in roller bottle incubations with live cells of strain TK-46(2) at day 7
944 shown floating underside the oil (A); *inset*, a magnified image of a MOS particle showing
945 associated oil droplets indicated by arrows. A MOS particle formed by live cells of strain TK-46(2)
946 viewed under the epifluorescence microscope (staining with acridine orange) shows these particles
947 to be foci where the cells (small green dots) and oil droplets (green blobs) are found concentrated
948 (B). Under the light microscope, these TK46(2)-formed MOS particles stained with Alcian Blue
949 (C), but not with Coomassie Brilliant Blue (D), and were found floating in a sea of oil droplets
950 (brown spheres), of which many were observed embedded within the amorphous matrix of the
951 particles. Partial emulsification of the oil into small oil droplets (0.5-2.0 mm) was observed in
952 incubations with live cells of *Pseudoalteromonas* TK105 (E). In live incubations with strain TK-8,
953 oil droplets were found heavily coated with the cells (F). Scale bars are 5 mm in A, E; 10 µm in B,
954 C, D, F.

955

956 **Figure 3.** Expansion 6.5 – 0.5 ppm of the ^{1}H NMR spectrum (400 MHz, 60 ºC in D_2O) of the TK-
957 46(2) polymer. Strong signals arising from monosaccharide residues are labelled as arising from
958 anomeric, ring, methylene and rhamnose methyl H6 protons. The residual partially deuterated water
959 signal (HOD) is at about 4.4 ppm and the acetone reference signal at 2.22 ppm. Peaks marked with
960 an asterisk are likely to arise from amino acid components, Asp, Glu and possibly Pro. B)
961 Expansion of the TOCSY spectrum of the same polysaccharide. Boxes indicate i) cross-peaks
962 between anomeric and ring protons of monosaccharide residues; ii) rhamnose H6 to ring proton
963 cross-peaks; iii) cross-peaks maybe attributable to proline.