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Enhanced crude oil biodegradative potential of natural phytoplankton-associated hydrocarbonoclastic bacteria

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Summary

Phytoplankton have been shown to harbour a diversity of hydrocarbonoclastic bacteria (HCB), yet it is not understood how these phytoplankton-associated HCB would respond in the event of an oil spill at sea. Here, we assess the diversity and dynamics of the bacterial community associated with a natural population of marine phytoplankton under oil spill-simulated conditions, and compare it to that of the free-living (non phytoplankton-associated) bacterial community. Whilst the crude oil severely impacted the phytoplankton population and was likely conducive to marine oil snow (MOS) formation, analysis of the MiSeq-derived 16S rRNA data revealed dramatic and differential shifts in the oil-amended communities that included blooms of recognized HCB (e.g. *Thalassospira*, *Cycloclasticus*), including putative novel phyla, as well as other groups with previously unqualified oil-degrading potential (*Olleya*, *Winogradskyella*, and members of the inconspicuous BD7-3 phylum). Notably, the oil biodegradation potential of the phytoplankton-associated community exceeded that of the free-living community, and it showed a preference to degrade substituted and non-substituted polycyclic aromatic hydrocarbons. Our study provides evidence of compartmentalization of hydrocarbon-degrading capacity in the marine water column, wherein HCB associated with phytoplankton are better tuned to degrading crude oil hydrocarbons than that by the community of planktonic free-living bacteria.

Introduction

Marine eukaryotic phytoplankton (micro-algae) contribute significantly to life on Earth by performing approximately half of global carbon fixation (Ducklow *et al.*, 2001) and producing almost half of the oxygen in the atmosphere (Field *et al.*, 1998). As protagonist members at the base of the food chain, they contribute a key role to the ecology of the marine ecosystem. Though still an area in a nascent state of our understanding, the interaction of phytoplankton with bacteria, which primarily occurs on the cell surface of phytoplankton cells (i.e. phycosphere), is thought to play importantly to their ecological success (Buchan *et al.*, 2014; Amin *et al.*, 2012; Amin *et al.*, 2015). Archaea may also play a pivotal role in phytoplankton dynamics and functioning, as evidenced in some recent reports (Orsi *et al.*, 2015; Needham and Fuhrman, 2016), but they have largely been ignored in this regard. Within an algal-bacterial community, the bacteria are likely to utilize algal exudates as carbon and energy sources (Bell and Mitchell, 1972; Myklestad, 1995), whereas the phytoplankton could benefit through bacterially mediated trace metal/nutrient bioavailability (McGenity et al., 2012). Amin and colleagues (2009), for example, showed a mutual sharing of iron and fixed carbon between several species of phytoplankton and bacteria, whereas Kazamia and colleagues (2012) reported the supply of bacterial-produced vitamin B12 to the eukaryote partner in exchange for fixed carbon. The fact that very few phytoplankton species can be maintained, or sub-cultured for long periods, in an axenic state (i.e. devoid of their bacterial symbionts) in the laboratory is testament to the important role that the associated bacterial, and possibly likely also archaeal, community plays in their overall success.

Members of three major phytoplankton lineages (dinoflagellates, diatoms, coccolithophores) have been found to harbour obligate and generalist hydrocarbonoclastic bacteria (HCB), including novel taxa of these organisms (Green *et al.*, 2006; Gutierrez *et al.*, 2012a,b; 2013b; 2014). The association of HCB with phytoplankton raises important questions with respect to their evolutionary genesis, ecology and response of these bacteria during a marine oil spill, and whilst the underlying basis for this remains to be defined, there is evidence suggesting that the enrichment of hydrocarbons

on phytoplankton cell surfaces plays an important role. By nature of their surface chemistry, phytoplankton cell surfaces have been shown to adsorb and accumulate polycyclic aromatic hydrocarbon (PAH) molecules (Mallet & Sarfou, 1964; Andelman and Suess 1970). Phytoplankton may also be a biogenic source of PAHs by synthesizing these compounds (Andelman and Suess, 1970; Gunnison and Alexander, 1975) and translocating them into the algal cell wall (Gunnison and Alexander, 1975; Zelibor *et al.*, 1988). Compared to the surrounding seawater, phytoplankton cell surfaces can thus become enriched with PAHs, and attract PAH-degrading bacteria to an available source of carbon and energy. Many phytoplankton also produce long-chain hydrocarbon-like compounds, such as alkenones (Marlowe *et al.*, 1984), and almost all produce the volatile hydrocarbon isoprene (Shaw *et al.*, 2010; Exton *et al.*, 2012) which could explain the occurrence of HCB found associated with these organisms. Whether through biogenic synthesis or adsorption of PAH molecules from the surrounding seawater, the cell surface of phytoplankton cells in the marine water column may act as a "hot spot" to which PAH-degrading bacteria exist, potentially in symbiotic relationship.

There are several factors about HCB living associated with phytoplankton that should be taken into account when these organisms come into contact with petrochemicals at sea. Oil contamination, particularly from large oil spills, is an important stressor that can significantly influence phytoplankton biomass. In general, field and laboratory studies have shown that crude oil concentrations up to 1.0 mg/L can stimulate phytoplankton growth – a phenomenon that may have an origin in ocean systems distinguished by the occurrence of natural hydrocarbon seeps on the sea floor where elevated surface chlorophyll concentrations have been reported (D'souza *et al.*, 2016). Higher crude oil concentrations, however, can cause anywhere from slight, severe to complete growth inhibition of phytoplankton (Nomura *et al.*, 2007; Adekunle *et al.*, 2010; Gilde and Pinckney, 2012; Gonzàlez *et al.*, 2013; Paul *et al.*, 2013; Ozhan *et al.*, 2014; Ozhan and Bargu, 2014a,b). The consequences of crude oil pollution at sea to phytoplankton must also take into account the associated bacterial community. Quite often, however, attention has focused to study one or the other

104 of these organisms. To take the Deepwater Horizon (DWH) oil spill as a recent example, the various
105 reports that had investigated the effects of the Macondo oil to phytoplankton populations in the Gulf
106 of Mexico during the spill did not focus on the associated bacterial community (Ozhan and Bargu,
107 2014a,b; Ozhan *et al*., 2014; Parsons *et al*., 2015), whereas the bacterial response had been
108 independently investigated in other studies (e.g. Bælum *et al*., 2012; Hazen *et al*., 2010; Gutierrez *et*
109 *al*., 2013c; Yang *et al*., 2016). These and a plethora of other studies that have employed seawater
110 sampling programs to study microbial population diversity and dynamics do not often employ
111 operational fractionation to tease apart the various microbial populations (based on size) that
112 constitute the water sample, hence leading to the misconception that HCB identified in any given
113 water column sample may have existed in a free-living state. It is not inconceivable that many of the
114 HCB identified in the plethora of reports in the literature describing their isolation, molecular
115 identification and/or dynamics, were likely physically attached to phytoplankton cells at the time of
116 their collection from the marine environment. Indeed, HCB associated with phytoplankton in marine
117 systems has largely been ignored.
118 A notable product from the interaction of oil with phytoplankton and their associated HCB
119 and other bacterial groups is the formation of marine oil snow (MOS). MOS was a distinguishing
120 feature to the DWH oil spill that is defined as mucilaginous organic matter with a "fluffy" or
121 gelatinous off-white appearance. Its formation has been reported to involve the interaction of
122 phytoplankton cells (Passow *et al.*, 2012), bacterial cells (Fu *et al.*, 2014; Arnosti *et al.*, 2015) and
123 extracellular polymeric substances (EPS) (Gutierrez *et al.*, 2013a; Passow *et al.*, 2012; Arnosti *et al.*,
124 2015; Ziervogel *et al*., 2012) with dissolved hydrocarbons and/or emulsified oil droplets (Passow *et*
125 *al.*, 2012; Ziervogel *et al*., 2012). MOS formation has in fact been reported to be more prominent
126 with bacterial-phytoplankton communities (Fu *et al.*, 2014; van Eenennaam *et al.*, 2016) compared to
127 when phytoplankton or bacterial cells are independently exposed to oil (van Eenennaam *et al.*, 2016).
128 EPS primarily produced by certain species of phytoplankton and bacteria can act as a 'sticky glue' in
129 binding particulates, such as microbial cells, in seawater (Wotton, 2004). Recent work has shown

that bacteria associated with phytoplankton contribute significantly to the bulk of EPS produced in seawater (van Eenennaam *et al.*, 2016), and that this bacterial-derived EPS is likely the main type of 'glue' in the formation of MOS (Gutierrez *et al.*, 2013; van Eenennaam *et al.*, 2016).

In light of increasing evidence showing the occurrence of HCB associated with marine phytoplankton, there is a paucity of knowledge that explains this algal-bacterial partnership and what role it plays during an oil spill at sea. The degradation of oil hydrocarbons has been demonstrated with artificial phytoplankton-bacterial consortia (Safanova *et al.*, 1999; Borde *et al.*, 2003; Muñoz *et al.*, 2003; Warshawsky *et al.*, 2007). Taking into account the intimate relationship that exists between phytoplankton and bacteria, including hydrocarbon-degraders, and the fact that enhanced degradation of hydrocarbons has been demonstrated when bacteria and phytoplankton coexist (e.g., Abed and Köster, 2005; Warshawsky *et al.*, 2007), studies aimed to investigate the microbial response to an oil spill at sea would be greatly informed by assessing the phytoplankton-bacterial community as a complete microbiological unit. In a recent study, Mishamandani *et al.* (2015) showed that the bacterial community associated with a laboratory culture of the marine diatom *Skeletonema costatum* is tuned to respond to and degrade aromatic hydrocarbons when challenged with crude oil. Transposing this investigation to the field, here we conducted an oil spill-simulated experiment with a natural phytoplankton-bacterial assemblage from the west coast of Scotland. The field sample was operationally fractioned to isolate the phytoplankton community from the free-living (non-associated) bacterial population, and both fractions then used to examine their response to crude oil. Sequencing of the 16S ribosomal RNA (rRNA) gene amplicons with the Illumina MiSeq technology was used to monitor the dynamics of the bacterial community and of HCB taxa in both oil-amended and non-amended incubations. Further, hydrocarbon analysis of oil extracts was used to assess the biodegradative potential of the community compared to the free-living bacterial fraction and infer their possible contribution to the overall biodegradation of the oil.

Results

To examine the microbial response and potential of phytoplankton-associated HCB communities from the west coast of Scotland on the biodegradation of crude oil, two oil enrichment treatments were set up. The first constituted the phytoplankton community (PHY) with its associated bacterial symbionts from Loch Creran surface water, whereas the second treatment comprised solely the free-living bacterial fraction (BAC) with phytoplankton removed by filtration; each of the PHY and BAC treatments were amended with Heidrun crude oil. A control treatment (CON) was also set up in the exact same way as treatment PHY, with the exception that the oil was not added.

Degradation of Heidrun crude oil

Student's T-tests confirmed that there were significant ($P < 0.05$) differences between the controls and PHY treatments for 5 of the hydrocarbon ratios analysed (Figure 1), specifically in reductions for both nC$_{17}$ and nC$_{18}$ aliphatic hydrocarbons and reductions in 2-methylnaphthalene and phenanthrene. In the BAC treatments, significant ($P < 0.05$) reductions compared to their controls in just a single parameter were indicative of biodegradation (i.e. 2-methylnaphthalene/2,6+2,7-dimethylnaphthalene [2MN/26+27DMN]) (Figure 1). These results suggested that biodegradation had occurred to a greater extent in the PHY (with phytoplankton) than in BAC (without phytoplankton) treatments for both aliphatic and aromatic hydrocarbons.

In the crude oil enrichment incubations with the phytoplankton (treatment PHY) or with the free-living bacterial fraction (treatment BAC), we determined the concentrations of various aliphatic and aromatic hydrocarbon species at the termination of the experiment (day 40). For this, the total volume of each of the 12 cultures that were designated for hydrocarbon analysis were extracted and analysed for total petroleum hydrocarbons (TPH) and their hydrocarbon composition, as described below. Compared with acid-inhibited controls, the concentrations of some hydrocarbon species were found to have significantly decreased ($P < 0.05$) after 40 days in the uninhibited (no acid treated)

incubations, and therefore was attributed to microbial biodegradation. Table 1 shows these hydrocarbons that were significantly biodegraded, and the percentage they were biodegraded compared to their concentrations in acid-inhibited controls. Of a total of 28 *n*-alkanes analysed (nC_8–nC_{35}), nine had been significantly degraded (i.e. nC_{20}–nC_{25}, nC_{27}, nC_{28} and nC_{31}) in treatment PHY, whereas eight *n*-alkanes (i.e. nC_{16}, nC_{17}, nC_{19}–nC_{24}) were significantly biodegraded in treatment BAC (Table 1). Some of the low-molecular-weight *n*-alkanes, such as nC_8, nC_9 and nC_{10}, were not detected in extracts from both the live and acid-inhibited incubations, suggesting they were likely lost by both evaporation and biodegradation over the course of the experiment. Similarly to the biodegradation of *n*-alkanes in these two treatments, more types of aromatic species (a total of 15 identified) were biodegraded in treatment PHY compared to twelve that were significantly biodegraded in treatment BAC (Table 1). The fifteen measured aromatic species biodegraded in treatment PHY included dibenzothiophene, 4-methyldibenzothiophene, C_{28}S-triaromatic steroid and several substituted species of naphthalene and phenanthrene. Overall, the BAC treatments displayed a preference for biodegrading the low-molecular-weight fraction of *n*-alkanes and aromatics compared to the PHY treatments which exhibited a preference for the higher-molecular-weight species.

Phytoplankton and bacterial cell population dynamics

The phytoplankton community in water samples from Loch Creran was comprised of a diversity of dinoflagellates and mainly diatoms, in particular species of *Thalassiosira, Chaetoceros* and *Skeletonema* (Table 2). Dominance was based on a cell count of $\geq 10^6$ cells l^{-1} for each species/genus identified. These diatoms are cosmopolitan and typically found to dominate the phytoplankton community in surface waters during the spring on the west coast of Scotland (Fehling *et al.*, 2006; Lappalainen and Tett, 2014).

To assess the phytoplankton community dynamics in response to crude oil, changes in Chlorophyll *a* (Chl *a*) were determined (Figure 2). In treatment CON without crude oil (incubations

C1, C2, C3), Chl *a* concentrations increased at a rate of 5.8 μg l^{-1} day^{-1}, from initial values of 7.1 ± 1.5 μg l^{-1} at day 0 and reached maximum levels of 54.0 ± 3.7 μg l^{-1} at day 8 (Figure 2C). Thereafter, Chl *a* concentrations decreased sharply at 8.3 μg l^{-1} day^{-1} within 3 days and continuing to decrease thereafter at an average rate of 1.0 μg l^{-1} day^{-1} and reaching 3.1 ± 2.9 μg l^{-1} at day 40. Conversely, no increase in Chl *a* concentrations were measured in the acid-inhibited controls as was expected (results not shown). The presence of crude oil in treatment PHY (incubations P1, P2, P3), however, had a marked effect on Chl *a* concentrations compared to the untreated control incubations (treatment CON). In the presence of the oil, phytoplankton growth was totally suppressed from the commencement of the experiment when the oil was added, with Chl *a* concentrations decreasing at a rate of 0.6 μg l^{-1} day^{-1}, from initial values of 8.5 ± 0.5 μg l^{-1} at day 0 to 0.7 ± 0.2 μg l^{-1} at day 14, and thereafter remaining at these levels until the termination of the experiment (Figure 2A). Microscopic examination of the culture liquid from treatment PHY did not reveal any live phytoplankton cells after 7 days following addition of the crude oil; intact cells showed signs of bleaching, and much of the phytoplankton population in this treatment had formed into flocs or aggregates (aka MOS) that persisted until the termination of the experiment. Similar observations were recorded in treatment CON, but much later (after day 20) in these incubations. Phytoplankton population dynamics via Chl *a* analysis were highly concordant with the abundance of 16S rRNA genes of chloroplasts (from our Illumina MiSeq analysis below) across the three treatments (results not shown).

Quantification of bacterial 16S rRNA genes in each of the three treatments (PHY, BAC, CON) was measured as a proxy for bacterial biomass; this method was used because accurate DAPI counts for prokaryotic abundance were, as explained above, impossible to obtain. As shown in Figure 2, bacterial 16S rRNA gene abundance across all three treatments decreased from day 0. The dynamic of bacterial gene abundance over the course of these 40-day experiments was similar between the phytoplankton (PHY; Figure 2A) and free-living bacterial (BAC; Figure 2B) treatments amended with oil, although it decreased approximately 25% more in the latter treatment. Conversely, the dynamic of bacterial 16S rRNA gene abundance in the phytoplankton control treatment without

234 oil (CON; Figure 2C) was markedly different, and showed a sharper drop in abundance within 5 days
235 compared to that in the PHY and BAC treatments. Thereafter, bacterial 16S rRNA gene abundance
236 increased over the next 3 days, then plateaued over the following 6 days, and then gradually
237 decreased until the termination of the experiment; by day 40 abundance values were one order of
238 magnitude lower compared to initial concentrations (Figure 2C). Visually the oil-treated incubations
239 with phytoplankton (treatment PHY) became gradually more turbid, albeit slightly, over the course
240 of the experiment when compared to the incubation with just the bacteria (treatment BAC) and, more
241 so, the untreated control (treatment CON). This increase in turbidity would be assumed indicative of
242 bacterial growth, possibly by the enrichment of HCB feeding on hydrocarbon components of the oil.
243 The gradual decrease in bacterial 16S rRNA genes in the PHY treatment (Figure 2A), however,
244 suggests that this increase in turbidity was less due to an increase in bacterial biomass, but likely
245 from the emulsification of the oil into dispersed oil droplets.
246
247 *MiSeq sequencing of bacterial community diversity and dynamics*
248 The diversity and response of the bacterial communities to crude oil was assessed in two replicates
249 for each of treatments PHY, BAC and CON using Illumina MiSeq technology. This was performed
250 at days 0, 8, 14, 24 and 40. Initially at day 0, the bacterial community in all the three treatments was
251 composed mainly of members within the *Gammaproteobacteria*, which was dominated by
252 *Pseudoalteromonadaceae* and *Vibrionaceae* – 71–78% combined contribution to the total 16S rRNA
253 gene sequence reads in each library – with minority representation from *Piscirickettsiaceae*,
254 *Oceanospirillales*, *Methylococcales* and several members of the Order *Alteromonadales* (additional
255 to *Pseudoalteromonadaceae*) that included *Psychromonadaceae*, *Colwelliaceae* and
256 *Alteromonadaceae* (Figure 3; Suppl. Table S1). The community also included minority
257 representation (<3%) from *Rhodobacterales* and *Flavobacteriales* of the classes *Alphaproteobacteria*
258 and *Bacteroidetes*, respectively, and of the Phylum *Verrucomicrobia*. By day 8, the abundance of the
259 *Pseudoalteromonadaceae* and *Vibrionaceae* dramatically decreased by at least 50% in all three

treatments (as much as 95% for *Pseudoalteromonadaceae* in treatment CON) compared to their initial abundance at day 0, and their abundance continued to decrease gradually thereafter until the termination of the experiment (day 40) reaching low (<0.6%) to undetectable levels. Over the duration of the experiment, the dominant members of the community in each of the three treatments (PHY, BAC, CON) belonged to the *Piscirickettsiaceae*, *Alteromonadaceae*, *Rhodobacterales* and *Flavobacteriales*.

Following addition of the oil to treatments PHY and BAC, the community changed markedly within 8 days relative to the control (treatment CON) with no oil added. At this time-point, the *Piscirickettsiaceae*, and to a lesser extent *Oceanospirillaceae* (incl. other *Oceanospirillales*), *Colwelliaceae* and *Flavobacteriales* showed a marked increase in abundance and were the major dominating groups in these oil-amended treatments. By days 14 and 24, the most dominant and enriched groups in treatments PHY and BAC were members of the *Piscirickettsiaceae* and to a lesser extent *Oceanospirillaceae* and *Colwelliaceae*. By day 40, the dominant groups in treatments PHY and BAC were members of the *Piscirickettsiaceae*. By day 40, the BD7-3 had gradually increased in abundance in treatment PHY over the duration of the experiment, whereas in treatment BAC members of the *Kiloniellales* became a dominant contributing group at day 40. The *Bacteroidetes* increased in abundance over the course of the experiment in only treatment CON. Overall, 20 operational taxonomic units (OTUs) were identified to have become enriched by the oil (Figure 4). Of the *Gammaproteobacteria*, these were OTU-6 (*Cycloclasticus*), OTU-7 (members of the *Colwelliaceae*), OTU-8 (other members of the *Priscirickettsiaceae*), OTU-9 (*Methylophaga*), OTU-10 (*Oleispira*), OTU-11 (*Marinomonas*), OTU-12 (*Alcanivorax*), OTU-13 (*Halomonas*), OTU-14 (*Shewanella*) and OTU-15 (*Psychromonas*); of the *Alphaproteobacteria*, OTU-1 (Order BD7-3), OTU-2 (*Hyphomonadaceae*), OTU-3 (other members of the *Rhodobacteraceae*), OTU-4 (*Thalassospira*), OTU-5 (*Phaeobacter*); of the *Betaproteobacteria*, OTU-19 (*Methylotenera*); of the *Bacteroidetes*, OTU-16 (other members of *Flavobacteriaceae*), OTU-17 (*Olleya*), OTU-18 (*Winogradskyella*); and of the *Verrucomicrobia*, OTU-20 (*Verrucomicrobium*). As shown in Figure

4, OTU-9 (*Methylophaga*) showed the strongest succession pattern in both treatments PHY and BAC, starting from day 8 and peaking in relative abundance by day14. The 16S rRNA gene sequence reads of these OTUs from this MiSeq survey were compared with related GenBank sequences, including sequences belonging to related HCB (Figure 5).

Discussion

The Loch Creran phytoplankton community was severely impacted when exposed to crude oil, as Chl *a* concentrations decreased immediately following exposure and reached near undetectable levels after 14 days. This is in concordance with the impact that the Macondo oil had upon the phytoplankton community in the Gulf of Mexico during the active phase of the DWH oil spill, which resulted in 85% reduction of phytoplankton abundance relative to baseline levels from previous years (Parsons *et al.*, 2015). The phytoplankton that survived the DWH perturbation represented a community with a reduced diversity that was dominated by diatoms and cyanobacteria (Parsons *et al.*, 2015). The Loch Creran phytoplankton community, however, appeared to have been decimated by the oil, as microscopic observations of sub-samples taken from the oil-treated incubations (PHY) did not reveal any live phytoplankton cells. Supporting this, Chl *a* concentrations had decreased by 92% of initial values by day 14 in these incubations, and we suspect that the very low residual Chl *a* levels measured after day 14 ($\leq$0.7 $\mu g\ l^{-1}$) may be attributed to non-decomposed extracellular or intracellular Chl *a* in dead or dying phytoplankton cells. Susceptibility of phytoplankton to oil varies among species, and whilst some species will thrive in oil-contaminated seawater, others will be detrimentally affected (González *et al.*, 2009; Adekunle *et al.*, 2010; Gilde and Pinckney 2012; Ozhan *et al.*, 2014). It has been suggested that diatoms are more sensitive to crude oil than other phytoplankton (Siron *et al.*, 1991) because the external silica frustule has a high affinity for absorbing hydrocarbons, wherein these chemicals accumulate and result in toxicity to the cells, such as by hindering sexual reproduction and auxospore formation (Kustenko, 1981). Since the

311 Loch Creran phytoplankton community was dominated by diatoms, this might explain its decimation
312 upon exposure to the Heidrun crude oil.

313 The lower basin of Loch Creran is highly exposed to wind, as was the case on the day of
314 sampling, which can cause mixing of the water column, including with the surficial sediment layer,
315 and potentially lead to replenishing the upper water column with nutrients. This nutrient input from
316 the sediment could explain the episodic increase and subsequent gradual decline of the
317 phytoplankton in the non-treated control incubations (CON). The bacterial population followed a
318 short-lived 'boom' and then 'bust' dynamics, which could have resulted from limited nutrient
319 concentrations in the Loch Creran water column. Nutrient limitation has also been shown to increase
320 the sensitivity of phytoplankton to crude oil (Ozhan and Bargu, 2014a; Karydis, 1981), which may in
321 part explain the dramatic decline in the phytoplankton population in the oil-amended incubations
322 (PHY). However, the extent to which phytoplankton are impacted by crude oil can largely depend on
323 the toxicological potency and concentration of the oil (Dunstan *et al.*, 1975; Vargo *et al.*, 1982; Bate
324 and Crafford, 1985; Huang *et al.*, 2011). Different phytoplankton species can have varying
325 tolerances to oil (Ozhan and Bargu, 2014a,b), and some may even be stimulated by it (Prouse *et al.*,
326 1976; Jung *et al.*, 2012; Parsons *et al.*, 2015). The most toxigenic hydrocarbons in crude oils are
327 those comprising the aromatic fraction – i.e. mono-aromatics and PAHs – which are recognized as
328 high-priority pollutants to the environment (Agency for Toxic Substances and Disease Registry,
329 2007; Boehm and Page, 2007). Toxicological effects of aromatic hydrocarbons to phytoplankton, in
330 particular the PAHs (Harrison *et al.*, 1986; Ozhan *et al.*, 2014), have been reported in several studies
331 (Ostgaard *et al.*, 1984a,b; Sargian *et al.*, 2005; Huang *et al.*, 2011); the toxicity of these chemicals to
332 phytoplankton have been reported at concentrations as low as 1 $\mu g\ L^{-1}$ (Ozhan *et al.*, 2014 and
333 references therein). Our analysis of the Heidrun crude oil used here showed it contained an aromatic
334 hydrocarbon content of 15% of TPH, comprised largely of relatively low-molecular-weight (e.g.
335 alkylated naphthalenes and phenanthrenes) PAHs that very likely contributed to the observed decline
336 in phytoplankton abundance in the oil-amended incubations.

Hydrocarbon analysis revealed that biodegradation of the crude oil was enhanced in the PHY incubations, as indicated by the the lower nC$_{17}$/pristane and the nC$_{18}$/phytane ratios in the PHY treatments relative to the acid inhibited controls, along with also lower ratios for 2-methylnaphthalene/1-methylnaphthalene, 2-methylnaphthalene/(2,6+2,7)-dimethylnaphthalene and phenanthrene/9-methylphenanthrene (P/9MP). On the other hand, the ratios for only 2-methylnaphthalene/(2,6+2,7)-dimethylnaphthalene were lower in the BAC treatments compared to their respective acid-inhibited controls. Interestingly, triaromatic steroids appeared to be significantly degraded in the PHY treatments, which was unexpected as these compounds are relatively recalcitrant to biodegradation (Radović *et al.*, 2014). However, their natural degradation has been reported in some laboratory- and field-based studies (Barakat *et al.*, 2002; Díez *et al.*, 2005; Radović *et al.*, 2014). The degradation of these cholestane-derivatives may be contributed by phytoplankton-associated bacteria encoding enzymes with a broad specificity for degrading cholesterols that can be found in the cell membranes of marine diatoms (Ponomarenko *et al.*, 2004). Notably, the phytoplankton-associated bacterial community of the PHY treatments exhibited an enhanced ability to degrade the oil, both in terms of the range and extent of hydrocarbon species degraded, as well as a preference for biodegrading higher-molecular-weight hydrocarbons, compared to that by the free-living community of the BAC treatments.

The formation of aggregated cells of phytoplankton and bacterial cells in the oil-amended incubations is reminiscent of the formation of marine oil snow (MOS) that was observed in profuse quantities in the Gulf of Mexico near the DWH spill site (Diercks *et al.*, 2010; Passow *et al.*, 2012). Of the major phytoplankton groups, diatoms reportedly can play a role in MOS formation (Passow *et al.*, 1994). Diatoms are a dominant group of phytoplankton communities in Scottish coastal waters (Leterme *et al.*, 2006; McQuatters-Gollop *et al.*, 2007), and they were a dominant group in the Loch Creran water column when we sampled in May 2013 – predominantly *Chaetoceros*, *Skeletonema* and *Thalassiosira*. Besides phytoplankton, there are several lines of evidence pointing to EPS-producing bacteria in also playing a protagonistic role in MOS formation. Firstly, EPS can take the form of

363 transparent exopolymer particles (TEP) that are recognized as a 'sticky glue', facilitating the
364 aggregation of microbial cells (phytoplankton, prokaryotes, fungi) to form marine snow (Alldredge
365 *et al.*, 1993; Passow *et al.*, 1994; Passow, 2002), or MOS in the presence of crude oil (Passow *et al.*,
366 2012; Ziervogel *et al.*, 2012; Gutierrez *et al.*, 2013a). Secondly, certain groups of bacteria, such as
367 *Halomonas* (Gutierrez *et al.*, 2013a), *Pseudoalteromonas* and *Alteromonas* (T. Gutierrez, M. Jones,
368 A.Teske, unpublished data), and *Colwellia* (Bælum *et al.*, 2012) have been shown to play a role in
369 the formation of MOS, specifically via their production of EPS. Thirdly, a recent report by van
370 Eenennaam *et al.* (2016) implicates EPS-producing bacteria associated with phytoplankton as major
371 contributors in MOS formation; even more so than EPS produced by non-associated bacteria and
372 axenic phytoplankton. In the only published report to-date to have examined the microbial
373 communities associated with MOS, Arnosti *et al.*, (2015) identified a diverse bacterial community on
374 MOS particles that was primarily composed of EPS-producing bacteria. Some of these taxa included
375 *Cycloclasticus* of the *Piscirickettsiaceae*, members within the *Bacteroidetes*, and diverse groups of
376 the *Alphaproteobacteria* (principally the *Roseobacter* clade) – organisms that were found enriched in
377 our oil-amended incubations with the Loch Creran phytoplankton-bacterial community. These
378 laboratory observations showing the formation of MOS might suggest that in the event of an oil spill
379 in coastal and offshore waters of Scotland, MOS will be expected to form. How much of it could be
380 formed in these waterways and what environmental impact this could have after its settlement to the
381 seafloor remains unknown and warrants investigation considering that large areas of the Scottish
382 coast contain diverse and sensitive benthic communities (Moore *et al.*, 1998; Bailey *et al.*, 2011).
383 The bacterial community in the treatments was initially dominated by members of the
384 *Vibrionaceae* and *Pseudoalteromonadaceae* – groups that are typically found associated with marine
385 phytoplankton (Buchan *et al.*, 2014) – and included putative oil-degrading taxa such as *Colwellia*,
386 although further work would be needed to confirm this. The control incubations, which did not
387 receive any oil (CON), reflected a bacterial community diversity and dynamic that is typical of a
388 phytoplankton bloom in the field (Buchan *et al.*, 2014), providing evidence to substantiate our

389 experimental set-up in simulating *in-situ* environmental conditions. Whilst bacterial abundance is
390 generally positively correlated with the abundance of phytoplankton during a bloom (Chang *et al.*,
391 2003), there is often an initial decoupling between the two, as is often seen by a dramatic initial
392 decrease and then subsequent increase in bacterial abundance (Riemann *et al.*, 2000). This was
393 indeed observed in the CON treatment, which comprised the phytoplankton community with its
394 bacterial symbionts, and although not fully understood it might result from bacterial competition
395 with phytoplankton for nutrients (Castberg *et al.*, 2001). Since the phytoplankton, and its bacterial
396 symbiont, populations did not recover after this initial decoupling event, nutrient limitation is likely
397 to explain for this, as mentioned above. The community in the CON treatment became progressively
398 dominated by members of the phylum *Bacteroidetes*, in particular the group flavobacteria which
399 became more abundant during the decay phase, as is typically observed in the field (Simon *et al.*,
400 1999; Riemann *et al.*, 2000; Pinhassi *et al.*, 2004).
401 While the crude oil had detrimentally impacted the phytoplankton community, analysis of the
402 MiSeq-derived 16S rRNA sequences revealed dramatic shifts in the free-living bacterial community
403 (BAC treatment) and community associated with the phytoplankton (PHY treatment). After addition
404 of the oil, the community in the PHY incubations shifted and became dominated initially by
405 members of the *Colwelliaceae* (predominantly *Colwellia*) and the *Oceanospirillaceae*, including
406 *Marinomonas* and *Alcanivorax*. *Alcanivorax* are often strongly selected for in oil-impacted
407 environments (Head *et al.*, 2006; Yakimov *et al.*, 2007) and have been found associated with marine
408 phytoplankton (Green *et al.*, 2004), whereas only a couple of studies have reported hydrocarbon-
409 degrading qualities for *Marinomonas* (Melcher *et al.*, 2002; Dong *et al.*, 2014). Hydrocarbon-
410 degrading *Colwellia* have also been reported to degrade hydrocarbons, such as strain RC25 that was
411 isolated from deep waters near the DWH spill site and for which there is evidence to suggest it may
412 have played a role in the formation of MOS during the spill (Bælum *et al.*, 2012). These organisms
413 were thus the first to respond upon exposure to Heidrun crude oil and, as indicated by our
414 hydrocarbon analysis, may have contributed importantly to the degradation of the aromatic

hydrocarbon fraction. The *Piscirickettsiaceae*, including *Methylophaga*, were also found significantly enriched in both the PHY and BAC incubations. This is intriguing from the point of view that *Methylophaga* have, until only recently, received little attention with respect to their role in the degradation of hydrocarbons. This is because members of this genus are recognized for their almost exclusive requirement for C_1 sources (e.g. methanol, methylamine, dimethylsulfide) as sole growth substrates, with the exception of some strains that are also capable of metabolising fructose (Janvier and Grimont, 1995). Some studies have reported the enrichment of *Methylophaga* exposed to oil or individual hydrocarbons in lab and field studies (Röling *et al.*, 2002; Yakimov *et al.*, 2005; Coulon *et al.*, 2007; Vila *et al.*, 2010; Techtmann *et al.*, 2015). Recent evidence showed that some members of this genus are indeed capable of utilising hydrocarbons as a sole source of carbon and energy (Mishamandani *et al.*, 2014). Intriguingly, these previous studies showed what appeared to be short-lived blooms of *Methylophaga* within the first few days after exposure to hydrocarbons, whereas the enrichment of these organisms in our PHY and BAC incubations showed them to have persisted until the termination of these experiments (day 40). Since *Methylophaga*, together with other members of the *Piscirickettsiaceae*, were the organisms most strongly selected for in both oil-amended incubations (PHY and BAC), we posit that these organisms may have contributed a role in the biodegradation of the Heidrun crude oil, particularly of the aromatic fraction. Since phytoplankton can produce large quantities of extracellular high-molecular-weight dissolved organic matter (DOM) that acts as a rich source of methylated sugars exploitable by bacterioplankton, such as methylotrophs (McCarren *et al*., 2010), *Methylophaga* may have also participated here in the recycling of phytoplankton-produced DOM. Furthermore, these enriched *Methylophaga*, represented by OTU-9, may represent novel species since they share ≤97% 16S rRNA gene sequence identity to their closest relative, the type strain *Methylophaga thiooxydans* strain DMS010^T (Boden *et al.*, 2010).

Other members that had become enriched by the oil relative to the untreated control (CON) included members of the order BD7-3 (OTU-1), *Rhodobacteraceae* (OTU-3), *Thalassospira* (OTU-

4), *Cycloclasticus* (OTU-6), *Oleispira* (OTU-10), *Halomonas* (OTU-13), *Flavobacteriaceae* (OTU-16), *Olleya* (OTU-17), *Winogradskyella* (OTU-18), *Methylotenera* (OTU-19) and *Verrucomicrobium* (OTU-20). Based on a BLASTn analysis of their 16S rRNA sequences, except for *Cycloclasticus* OUT-6, these organisms may represent novel species since they each shared ≤99% identity to closest type strains; in the case of BD7-3 (OTU-1) and *Verrucomicrobium* (OTU-20), the highest sequence match to a type strain was 88% and 91%, respectively. Of these taxa, *Thalassospira* and *Halomonas* contain members with reported hydrocarbon-degrading qualities (Zhao *et al.*, 2010; Gutierrez *et al.*, 2013a and references therein), and *Oleispira* is a genus comprising members of obligate hydrocarbon-degraders with a preference for utilizing straight-chain aliphatics (Head *et al.*, 2006; Yakimov *et al.*, 2007). The family *Rodobacteraceae* includes the *Roseobacter* clade, which is commonly found in high abundance during algal blooms (González *et al*., 2000). Its enrichment in the PHY incubations, albeit moderately greater than in the CON incubations, may be attributed to these organisms encoding multiple ring-cleaving pathways that participate in the degradation of monocyclic and PAHs (Moran *et al*., 2007). Notably, members of the order BD7-3, like the *Piscirickettsiaceae* (incl. *Methylophaga*) and *Colwelliaceae*, were strongly enriched in the PHY incubations amended with oil. Members of this order are inconspicuous by the very fact that they are poorly represented in 16S rRNA gene databases and their distribution and ecology is practically unknown. To the best of our knowledge, no reports have described any member of the BD7-3 to degrade hydrocarbons, and our results provide the first evidence to suggest that these organisms might encode this phenotype, or play an indirect role in hydrocarbon biodegradation. The *Flavobacteriaceae* contain members with hydrocarbon-degrading qualities, including *Arenibacter* which is a genus comprising members with the ability to degrade PAHs (Gutierrez *et al.*, 2014). *Arenibacter* associated with diatoms have been shown to become enriched by crude oil (Mishamandani *et al.*, 2015), and although members of *Shewanella* are not commonly associated with oil spills, a hydrocarbon-degrading species of this genus has been described that originated from Antarctic waters (Gentile *et al.*, 2003). All the other taxa that were enriched by the oil (i.e. *Olleya*,

467 *Winogradskyella*, *Methylotenera*, *Verrucomicrobium*) have, to the best of our knowledge, no
468 representatives that have been described to degrade hydrocarbons.

469 This study represents the first investigation on the response of a natural phytoplankton-
470 bacterial assemblage to crude oil with a focus in teasing apart and comparing the dynamic response
471 of the phytoplankton-associated bacterial community to that which is free-living, including a
472 paralleled analysis of the hydrocarbons degraded by these communities. Our findings show that the
473 associated bacterial community contributed an important role in the degradation of the more toxic
474 aromatic hydrocarbon components of crude oil. Following from our previous study where we
475 showed this preferential degradation of aromatic hydrocarbons in crude oil by the bacterial
476 community associated with the cosmopolitan marine diatom *Skeletonema costatum* (Mishamanadani
477 *et al.*, 2015), our work highlights phytoplankton as a natural biotope in the ocean that harbour a
478 diversity of HCB. Our work has revealed the presence of novel taxa associated with marine
479 phytoplankton that respond upon exposure to oil, notably members of the order BD7-3,
480 *Methylotenera* and *Verrucomicrobium*, that may be putative hydrocarbon-degraders. The prominence
481 of HCB with phytoplankton could be described as a hallmark of phytoplankton, standing at the ready
482 as sentinels in the water column to respond and help purge the marine water column in the event of
483 petrochemical inputs. Considering the close interactions between phytoplankton and microbial
484 communities, and including the apparent ubiquitous representation of hydrocarbon-degraders to the
485 associated bacterial community (McGenity *et al.*, 2012), future oil biodegradation and
486 bioremediation studies should therefore not ignore these associations.
487

488 ## Experimental Procedures

489 *Field sample collection and crude oil enrichment set-up*

490 Loch Creran is a 12.8 km long fjordic sea loch (1.3 km average width; 13.5 m average depth; 49 m
491 max. depth) that is located on the west coast of Scotland and receives waters from the open sea.
492 During a sampling operation aboard *RV Serpula* on 24 May 2013 to a sampling station in the lower

493 basin of Loch Creran (56º30.820N, 5º22.817W), we trawled a phytoplankton net (50-60 μm mesh
494 size) near the sea surface (1–2 m depth) for several minutes to collect the phytoplankton community;
495 we also collected ca. 10 litres of seawater into a pre-sterilized 10-L polypropylene carboy. The lower
496 basin of Loch Creran (56º30.820N, 5º22.817W) was chosen here for investigating the response of
497 algal-bacterial communities to crude oil as it contains relatively high levels of phytoplankton for
498 western Scottish coastal waters (Tett and Edwards, 2002). The seawater and phytoplankton net
499 samples were passaged through a 125-μm metal mesh filter to remove grazers, then stored at 4°C and
500 used within 24 hours for the preparation of the various experiments described below. Sub-samples
501 were sent to the Scottish Environmental Protection Agency (SEPA) for microscopic identification of
502 the phytoplankton taxa and their abundance in the collected samples.
503 In order to examine the microbial response and potential of phytoplankton-associated HCB
504 communities on the biodegradation of crude oil, two oil enrichment treatments, PHY and BAC, were
505 set up. Treatment PHY constituted the phytoplankton with its associated bacterial community from
506 the Loch Creran surface water sample. The inoculum for this treatment was prepared by adding 500
507 mL of the washed phytoplankton net trawl to 10 L of filtered (0.2 μm) seawater. Treatment BAC
508 comprised solely the free-living bacterial fraction, the inoculum for which was prepared by passing
509 ca. 1 L of the Loch Creran water through 2-μm (Isopore) filters to remove phytoplankton cells. Each
510 of the two treatments (PHY and BAC) was conducted in triplicate using autoclaved 1-L Erlenmeyer
511 flasks, which were filled with 350 ml of the respective inoculum prepared as described above. To
512 each of the triplicate flasks for treatment PHY (designated P1, P2, P3) and for treatment BAC
513 (designated B1, B2, B3) were amended with 0.2 μm-sterilised Heidrun crude oil to a final oil slick
514 content of ca. 1% (v/v) – an amount that has been used to simulate laboratory-controlled oil
515 enrichments (Piehler *et al.*, 1999). Heidrun crude oil (Statoil) was sourced from the Norwegian
516 Sector of the North Sea and is classed as a light-to-medium density, low sulfur crude oil. Heidrun
517 crude oils can vary with respect to their composition, which largely depends on their extent of
518 weathering in the field from where they are sourced. Generally, they consist of approximately 46%

519 naphthenes, 38% aromatics, 15% paraffins and other compounds, with varying proportions of *n*-
520 alkanes that can range from undetectable to low percent content of TPH since this fraction of
521 hydrocarbons is often the most susceptible to biodegradative processes (Karlsen *et al.*, 1995). GC-
522 MS analysis of the Heidrun crude oil used in our study revealed it contained C_8–C_{35} *n*-alkanes that
523 collectively contributed ca. 4%, with a higher proportion of aromatics at ca. 15% of TPH.
524 Ratios of *n*-alkanes to acyclic isoprenoid hydrocarbons (*n*C17/pristane and *n*C18/phytane)
525 were used as convenient indicators of biological degradation, due to the recalcitrance imparted by the
526 branched structure of the isoprenoid biomarkers (Sauer and Boehm, 1991; Papazova and Pavlova,
527 1999; Dawson et al., 2013). Similarly for aromatic hydrocarbon analysis, this was done for 7 ratios
528 indicative of biodegradation (naphthalene/2-methylnaphthalene; 2-methylnaphthalene/1-
529 methylnaphthalene; 2-ethylnaphthalene/2,6+2,7-dimethylnaphthalene; 2-methylnaphthalene/2,6+2,7-
530 dimethylnaphthalene; phenanthrene/9-methylphenanthrene; 3+2-methylphenanthrene/9+1-
531 methylphenanthrene; 3-methylphenanthrene/9-methylphenanthrene).
532 A third treatment (treatment CON) was set up in the exact same way as treatment PHY, with
533 the exception that the oil was not added in order to serve as untreated controls (designated C1, C2,
534 C3). All nine flasks were incubated in a temperature-controlled 15°C illuminated incubator with a
535 12:12 light/dark cycle and at a photon flux density of ca. 100 μmol s^{-1} m^{-2}. Samples for micro-algal
536 and bacterial counts were taken at days 0, 2, 5, 8, 11, 14, 24 and 40. Samples for molecular analysis
537 were taken at days 0, 8, 14, 24 and 40. Prior to sampling, the contents of each flask was stirred by a
538 magnetic stir bar that had been placed inside each flask at the time they had been autoclaved.
539 In order to analyze for changes in the composition of the oil due to biodegradation, an
540 additional six flasks were prepared in the same way as for treatment PHY. Of these six flasks, three
541 were treated with 85% phosphoric acid to a final concentration of 3% to serve as the acid-inhibited
542 controls. An additional six flasks were prepared in the same way as for treatment BAC, of which
543 three were treated in the same way with 85% phosphoric acid. All twelve flasks (six uninhibited and
544 six acid-treated) were incubated in parallel together with the flasks above in the temperature-

545 controlled illuminated incubator. At the termination of the experiment (day 40), these twelve flasks
546 were extracted for total petroleum hydrocarbons (TPH) and subsequent analysis for individual
547 hydrocarbon constituents by gas chromatography/mass spectrometry (GC-MS), as detailed below.
548
549 *Hydrocarbon analysis*
550 Each flask was sacrificed at specified time points for extraction of TPH using
551 dichloromethane (DCM) at an oil/water mix to DCM ratio of 1:2. The DCM fraction was removed
552 and the oil/water mix re-extracted an additional 3 times. The extracted oil sample was then diluted
553 with DCM to ca. 5ml and dried by the addition of a small amount of anhydrous sodium sulphate. The
554 sample was removed from the sodium sulphate using a pipette. The sodium sulphate was washed 4
555 times with a small amount of DCM and the washings were combined with the dilute oil sample.
556 Sample volume was reduced to ca. 2-3ml, transferred to a measuring cylinder and diluted to 5ml. An
557 aliquot of known volume was removed, evaporated to dryness and weighed. The gravimetric data
558 were used to calculate the original sample weight and the weight of oil remaining.
559 A known aliquot corresponding to ca. 30mg was taken from the remaining sample and
560 transferred to a 10ml vial. An aliquot of the reference oil was weighed directly into a vial and diluted
561 with ca. 0.3ml DCM. Squalane and 1,1'-binaphthyl were added as surrogate standards at ca. 0.5%
562 and 0.05% by weight of the oil, respectively. A procedural blank including the standards was also
563 prepared. One sample was analysed in triplicate and the reference oil was analysed in duplicate.
564 A chromatographic column was prepared using silica topped with alumina. Both sorbents
565 were pre-extracted with DCM and activated at 120°C prior to use. The sorbents were introduced as
566 slurries in petroleum ether (b.p. range 40-60°C). The sample (sorbed to ~3 g alumina) was applied to
567 the top of the column. The total hydrocarbon (TPH) fraction was eluted with 50ml petroleum ether
568 followed by 70ml petroleum ether/DCM (2:5). Solvent was reduced to ca. 2ml using a Heidolph
569 rotary evaporator. The sample was transferred to a vial and diluted to 3.4ml, and an aliquot was
570 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 surrogate standard squalane were obtained. The total hydrocarbon content was calculated using the manually integrated area under the whole chromatogram, drawing a horizontal baseline from the start of the solvent peak to the end of the acquisition. The corresponding total area for the procedural blank (which also contained the surrogate 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. 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) 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, initial inlet pressure of 50kPa, split at 30 ml/min). 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.

Concentrations of aliphatic and aromatic hydrocarbon species/groups that were biodegraded after 40 days were 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 analysed between the treatments.

Extraction of DNA

Cell biomass from samples (5 ml) taken at each sampling point from treatments PHY, BAC and CON was collected by filtration using a glass vacuum filtration system (Millipore) with 25 mm polycarbonate membrane filters (0.2 µm pore size; Isopore) and the filters stored at -20ºC. The polycarbonate membranes were placed into 1.5 ml Eppendorf tubes containing 200 µl reaction buffer (10mM Tris; 1 mM EDTA; 0.5 % (w/v) SDS; 50 µg ml^{-1} proteinase K). Tubes were gently vortexed and incubated for 30 minutes at room temperature. Filters were then dissolved with the addition of 200 µl of pH 8 equilibrated phenol:chloroform:isoamyl alcohol (25:24:1) for 5 minutes. Reactions were centrifuged (10,000 rpm; 5 min) and DNA in the aqueous top phase was transferred to a clean tube. TE (200 µl) was added to the original tube and centrifuged a second time to capture the remaining nucleic acid. The aqueous phases were combined and nucleic acid was precipitated with 10% NaCl and 2.5x volumes of *iso*-propanol for 30 minutes on ice. The DNA was recovered by centrifugation (17,000 rpm; 10 min), washed with 70% ethanol, dried and then resuspended in 50 µl of TE buffer. Purified DNA was stored at -20ºC for subsequent quantification and molecular analysis. For quantitation of DNA, a NanoDrop ND-3300 fluorospectrometer (Labtech, East Sussex, UK) and the Quant-iT Picogreen double-stranded DNA (dsDNA) kit (Invitrogen, Carlsbad, CA, USA) were used.

Quantification of the bacterial population and phytoplankton by real-time PCR and Chl a *measurements*

623 Samples (2 ml) for Chl *a* determinations were extracted using a modified version of EPA Method
624 445 (Arar and Collins, 1997). For this, each 2-ml sample was filtered through a 25 mm Glass Fiber
625 Filter (GF/F) and then placed into 10 ml of 90% acetone. Samples were sonicated in an ice water
626 bath for 10 min in the dark and then maintained at -20°C for ca. 20 hours. The samples were then
627 centrifuged and the supernatant fractions transferred to clean tubes and allowed to equilibrate in the
628 dark at room temperature prior to fluorometric analysis on a Turner Trilogy Fluorometer (Turner
629 Designs, CA 94085) using an excitation wavelength of 485 nm and emission of 685 nm. Chl *a*
630 concentrations were calculated from a standard curve constructed from serial dilutions of a Chl *a*
631 extract (Turner Designs, CA 94085) as per the method of Welshemeyer (1994).
632 To quantify the prokaryotic (bacteria and archaea) response to crude oil, we initially used the
633 DAPI (4'6-diamidino-2-phenylindole) staining technique. However, this was not pursued further
634 because we observed clusters of prokaryotic cells attached to live and dead/dying phytoplankton
635 cells, including aggregates of these, which made it impossible to provide an accurate measure of
636 prokaryotic cell counts. We therefore decided to focus on the bacterial community since to-date no
637 archaeal representative has been identified with the ability to degrade hydrocarbons. For this, we
638 quantified the abundance of bacterial 16S rRNA genes, as a proxy for bacterial biomass, by real-time
639 quantitative PCR (qPCR) using bacterial primers 341f (Muyzer *et al.*, 1993) and 518r (Øvreås *et al.*,
640 1997). Single reactions were performed on each triplicate DNA extraction from triplicate samples
641 collected at each sampling time point. The template for the construction of the respective standard
642 curve for qPCR was a PCR amplicon of *E. coli* 16S rRNA gene that was amplified with *E. coli*
643 primers ECP79f (5'-GAAGCTTGCTTCTTTGCT-3') and ECR620r (5'-
644 GAGCCCGGGGATTTCACA-3'). An annealing temperature of 55 °C was used for the qPCR
645 programme employing these primers.
646
647 *Barcoded amplicon metagenomic sequencing and analysis*

Barcoded 16S rRNA gene MiSeq was used to analyse the bacterial community in the purified DNA extracts from samples collected at days 0 (prior to oil amendment), 8, 14 and 40. Almost full length 16S rRNA sequences were amplified using the 27f (Wilmotte *et al.*, 1993) and 1492r (Lane, 1991) primers (20 µM) in 50 µl reactions containing 45 µl Supermix (IDT Invitrogen) and 1 µl of nucleic acid extract. We performed an initial extension of 1 minute at 94 °C followed by 35 PCR cycles (45 seconds at 94 °C; 45 seconds at 45 °C; 1 minute at 72 °C) and a final denaturation stage of 5 minutes at 72 °C. PCR products were then cleaned by addition of 5 µl FastAP (1U μl^{-1}) and 7.5 µl Exonuclease I (1U μl^{-1}) (Life Technologies) (45 minutes at 37 °C; 15 minutes at 85 °C). A second round of PCR targeting the hypervariable V3 and V4 regions was performed using MiSeq forward (5' AATGATACGGCGACCACCGAGATCTACAC <8-nt i5 barcode> TATGGTAATT GT ACWCCTRCGGGWGGCWG3') and reverse (5'-CAAGCAGAAGACGGCATACGAGAT <8-nt i7 barcode> AGTCAGTCAG CC ACCAGGGTATCTAAKCCTG 3') primers (20 µM). Primers were added to a tube containing 5 µl of cleaned PCR product, MyTaq polymerase (5 U), MyTaq reaction buffer (1x) and DMSO (1.5 %) (total of 20 µl reaction). For this round we performed an initial extension of 3 minutes at 95 °C followed by 28 PCR cycles (15 seconds at 95 °C; 15 seconds at 55 °C; 1 minute at 72 °C) and a final denaturation stage of 5 minutes at 72 °C. Once again the PCR product was cleaned by addition of 2 µl FastAP (1U L^{-1}) and 3 µl Exonuclease I (1U L^{-1}) (45 minutes at 37 °C; 15 minutes at 85 °C). The expected length of the amplicon after the second round was ~ 450 mer.

Samples were sequenced via the Illumina MiSeq platform at the University of Liverpool Centre for Genomic Research where a final 8 cycles of PCR were performed with Illumina Nextera XT forward and reverse primers containing a compatible adapter and a 2 bp linker sequence. Raw Illumina fastq forward and reverse files were merged via Pandaseq and sequences smaller than 400 mer were trimmed using Prinseq (~80% of sequences were conserved), producing a total of 3,140,774 reads. QIIME software was used to cluster the sequences into operational taxonomic units (OTUs) at 97% similarity. Representative sequences were blasted and the GreenGenes database was

674 used to assign taxonomy to the representative strain/OTU cluster. Data were normalised by
675 expressing the number of sequences in each OTU cluster as a percentage of total sequences for each
676 sample library. Chloroplast sequences were deleted and percentage abundances of bacterial 16S
677 rRNA gene sequences were adjusted accordingly. Representative OTUs of unknown phylogenetic
678 classification were checked for known sequences using RDP and BLASTn. A heatmap was produced
679 manually using Microsoft Excel to show all OTUs, with a relative abundance of >1%, that had
680 become enriched in the Heidrun crude oil incubations. Sequence data were submitted to MG-RAST
681 under ID number mgm4742856.3.
682
683 *Phylogenetic tree*
684 Representative 16S rRNA gene sequences of OTUs representing putative and recognized members
685 of hydrocarbon degrading taxa identified by MiSeq analysis were aligned using CLUSTAL_X
686 programme (Thompson *et al.*, 1994). Sequences and type strains with the highest sequence similarity
687 from GenBank were also used for tree construction. A neighbour-joining tree was constructed with
688 TREEVIEW (WIN32) version 1.5.2 (Page, 1996) and bootstrap replication (n=1000), and
689 *Methanobacterium aarhusense* (AY386124), *Methanobacterium flexile* (NR116276) and
690 *Methanobacterium paludis* (NR133895) were used as the outgroup.
691
692 *Nucleotide sequence accession numbers*
693 GenBank accession numbers for the representative OTUs that were identified as enriched by crude
694 oil are as follows: BD7-3 OTU-1 (KY962625), *Hyphomonadaceae* OTU-2 (KY962626),
695 *Rhodobacteraceae* OTU-3 (KY962627), *Thalassospira* OTU-4 (KY962628), *Phaeobacter* OTU-5
696 (KY962629), *Cycloclasticus* OTU-6 (KY962630), *Colwelliaceae* OTU-7 (KY962631),
697 *Piscirickettsiaceae* OTU-8 (KY962632), *Methylophaga* OTU-9 (KY962633), *Oleispira* OTU-10
698 (KY962634), *Marinomonas* OTU-11 (KY962635), *Alcanivorax* OTU-12 (KY962636), *Halomonas*
699 OTU-13 (KY962637), *Shewanella* OTU-14 (KY962638), *Psychromonas* OTU-15 (KY962639),

Flavobacteriaceae OTU-16 (KY962640), *Olleya* OTU-17 (KY962641), *Winogradskyella* OTU-18 (KY962642), *Methylotenera* OTU-19 (KY962643) and *Verrucomicrobium* OTU-20 (KY962644).

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Table and Figure legends

Table 1. Hydrocarbons biodegraded during enrichment of the phytoplankton (treatment PHY) or free-living bacterial (treatment BAC) fractions with Heidrun crude oil.

Table 2. Eukaryotic phytoplankton taxa for which genus and/or species designations could be attributed that were identified at high ($\geq 10^6$ cells l^{-1}), moderate ($\geq 2.0 \times 10^4$ to $\leq 10^6$ cells l^{-1}) or low (≥ 1 to $\leq 2.0 \times 10^4$ cells l^{-1}) abundance at Loch Creran during the spring of 2013. The class of each phytoplankton taxon is shown.

Figure 1. Differences in hydrocarbon ratios (significant results only, Student's T-test, $P < 0.05$) comparing live treatments (BAC or PHY) to their respective acidified controls for five characteristic

parameters indicative of biodegradation: 2-methylnaphthalene/1-methylnaphthalene (2MN/1MN), 2-methylnaphthalene/(2,6+2,7)-dimethylnaphthalene (2MN/26+27DMN), phenanthrene/9-methylphenanthrene (P/9MP), *n*C17/pristane (nC17/pr), *n*C18/phytane (nC18/phy). Values are averages of triplicate incubations. Error bars show standard error. The dashed line shows the division between PHY and BAC treatments.

Figure 2. Chlorophyll *a* concentrations (*solid circles*) and bacterial 16S rRNA genes (*open circles*) in incubations of the phytoplankton (treatment PHY) or bacterial (treatment BAC) community amended with Heidrun crude oil (respectively, graphs A and B) or the phytoplankton without any added oil (treatment CON; graph C). Each point represents the average and standard deviation of triplicate Chl *a* or qPCR measurements from independent incubations. Some error bars are smaller than the symbol.

Figure 3. Composition of bacterial 16S rRNA gene MiSeq reads from incubations of the phytoplankton (treatment PHY) and free-living bacterial (treatment BAC) community with Heidrun crude oil, and of the phytoplankton without oil (treatment CON). The relative abundance of taxa present at ≥1% relative abundance is shown. Sequences were classified to family-level taxonomy when possible and otherwise a higher-level classification is shown.

Figure 4. Heatmap of all OTUs enriched in the oil-amended incubations (treatments PHY and BAC) compared to the controls (treatment CON). OTUs were considered enriched if there was a mean increase of at least 1% relative abundance (e.g. shift from 1% to 2%) in at least one time point for replicate incubations, and if the difference was statistically significant ($P < 0.05$). Colour key indicates square-root normalized relative abundance (%). A strong succession pattern in the oil-amended treatments (P and B) was apparent for members of the *Piscirickettsiaceae*: OTU-9 (*Methylophaga*) peaked in relative abundance on day 40 in treatment P, and at day 14 in treatment B;

and OTU-8 (other *Piscirickettsiaceae*) peaked in relative abundance on day 14 in treatment P.

**Betaproteobacteria*, ***Verrucomicrobia*

Figure 5. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences (>1,200 bp), showing the 20 OTUs enriched (in bold) in the oil-amended incubations alongside representatives of related taxa. Filled circles indicate nodes with bootstrap values (1,000 bootstrap replications) greater than 90%; open circles indicate bootstrap values greater than 60%. GenBank accession numbers are shown in parentheses. The scale bar, indicates the number of substitutions per site.