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COREXIT 9500 enhances oil biodegradation and changes microbial community structure of oil-enriched microcosms

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Running Title: Effect of COREXIT 9500 on biodegradation

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ABSTRACT

To better understand the impact of COREXIT 9500 on the structure and activity levels of hydrocarbon degrading microbial communities, we analyzed next-generation 16S rRNA gene sequencing libraries of hydrocarbon enrichments grown at 5 and 25 °C by using both DNA and RNA extracts as sequencing templates. Oil biodegradation patterns in both 5 and 25°C enrichments were consistent with those reported in the literature (i.e., aliphatics were degraded faster than aromatics). A slight increase in biodegradation was observed in the presence of COREXIT at both temperatures. Differences in community structure were observed between treatment conditions in the DNA-based libraries. The 25 °C consortia was dominated by *Vibrio*, *Pseudoidiomarina*, *Marinobacter*,

Alcanivorax, and *Thallassospira* species, while the 5 °C consortia were dominated by several genera of *Flavobacteria*, *Alcanivorax* and *Oleispira*. Most these genera have been linked to hydrocarbon degradation and have been observed after oil spills. *Colwellia* and *Cycloclasticus*, known aromatic degraders, were also found in these enrichments. The addition of COREXIT did not have an effect on the active bacteria community structure of the 5 °C consortia, while at 25 °C a decrease in the relative abundance of *Marinobacter* was observed. At 25 °C, *Thallasospira*, *Marinobacter*, and *Pseudoidiomarina* were present at higher relative abundance in the RNA than DNA libraries suggesting that they were active in degradation. Similarly, *Oleispira* was greatly stimulated by the addition of oil at 5 °C.

IMPORTANCE

While COREXIT 9500 is widely applied after oil spills for its reported dispersing activity, there is still debate on the effectiveness on enhancing oil biodegradation and its potential toxic effect on oil-degrading microbial communities. The results of this study provide some insights on the microbial dynamics of hydrocarbon degrading bacterial populations in the presence of COREXIT 9500. Operational Taxonomic Unit (OTU) analyses indicated that several OTUs were inhibited by the addition of COREXIT. Conversely, a number of OTUs were stimulated by the addition of the dispersant, many of which were identified as known hydrocarbon degrading bacteria. The results highlight the value of using RNA-based methods to further understand the impact of dispersant on the overall activity of different hydrocarbon degrading bacterial groups.

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63 **Introduction**

64 Diverse marine microbes have the ability to degrade crude oil under both aerobic and
65 anaerobic conditions (1, 2). Crude oil is a complex mixture of hydrocarbons, with
66 constituents including both branched and straight chain alkanes as well as polycyclic
67 aromatic hydrocarbons (PAHs). In addition to natural inputs of oil into the marine
68 environment(3), oil has been released via accidental oil spills. These oil spills have the
69 potential to drastically impact ecosystems and result in long-term environmental changes
70 (4, 5). Various strategies have been employed to aid in the cleanup of oil spills, including
71 dispersant application (6). Chemical dispersants contain a mixture of chemicals designed
72 to break up an oil slick by reducing the interfacial surface tension of the oil, causing it to
73 form micron-sized droplets. These droplets can then be diluted in the water column
74 preventing the formation of a surface slick. Indigenous oil-degrading microbes are
75 believed to degrade these dispersed oil droplets, with smaller droplets being more rapidly
76 degraded (7).

77 During the *Deepwater Horizon* oil spill 2.1 million gallons of the chemical
78 dispersant COREXIT 9500 were applied at both the surface and at the seafloor (8).
79 There is debate about the effect of COREXIT 9500 on both the rates of oil
80 biodegradation as well as its effects on microbial community composition. Several
81 reports from diverse marine locations have demonstrated that the addition of COREXIT
82 9500 enhances oil biodegradation (9,10,7,11,12). Other studies have shown a neutral or
83 inhibitory effect of COREXIT 9500 addition on microbial activity (13-15). A number of
84 factors could explain these discrepancies including differences in experimental set up,

length of experiment, water source (including handling and storage) and concentrations of oil and dispersants. These contrary findings underscore the need for more in-depth analysis of the effect of COREXIT 9500 on oil biodegradation.

There are also differing reports of the impact of COREXIT on the microbial community composition. One recent study used high-throughput 16S rRNA gene sequencing to investigate the effect of oil and dispersant on microbial community structure (9). They demonstrated that many of the same taxa dominated in the oil-only and oil plus dispersant treatments. The minimal effect of COREXIT 9500 on community structure in Baelum *et al* (2012) is underscored by the very high COREXIT 9500 concentration used in their study. While a 1:25 dispersant to oil ratio was the target ratio for subsea dispersant application during the *Deepwater Horizon* oil spill (10, 16), Baelum et al (2012) used a dispersant to oil ratio of 1:1.6. Another recent study investigated the impact of COREXIT 9500 on both biodegradation of the water accessible fraction (WAF) of oil as well the microbial community structure (15). The authors conclude that COREXIT 9500 addition resulted in a distinct community of oil degraders.

The majority of previous studies examining the effect of COREXIT 9500 addition on oil biodegradation have focused on South Louisiana Crude oils. Alaska North Slope (ANS) crude oil is another important oil type that has been focus of oil biodegradation studies. COREXIT 9500 has been shown to effectively disperse ANS in wave tank experiments (18) as well as enhance biodegradation of ANS crude oil by Chukchi Sea microbes in microcosms incubated at -1 °C (10). However, the impact of COREXIT 9500 on the composition of microbial communities degrading ANS oil has not been reported. We investigated the effect of COREXIT 9500 on biodegradation of ANS crude

oil and the impact of COREXIT addition on microbial community structure of two hydrocarbon degrading consortia. Next-generation sequencing of the 16S rRNA gene using both DNA and RNA extracts as sequencing targets was used to study the microbial community composition. By comparing the results on both the DNA and RNA level we determined the effect of COREXIT 9500 on the total bacteria community structure (DNA) as well as the active bacterial fraction (RNA). The goal of this present study was better understand the effects of COREXIT 9500 on oil biodegradation, and to seek to reconcile the contrary findings regarding the impacts of COREXIT 9500 addition on oil biodegrading and community structure changes.

Experimental Methods

Microcosm set-up

Two different enrichment cultures generate from samples collected nearby the site of *Deepwater Horizon* oil spill (19). One culture was derived from a surface sample and the other was derived from a deep-water sample. These enrichments were grown at temperatures that reflect those from where the samples were originally collected. The methods by which these enrichments were derived can be found elsewhere (17). Briefly, the enrichments were originally grown at 5 °C (referred to as the cryo enrichment) or 25 °C (referred to as the meso enrichment) on GP2 medium (pH 7.5) (20) amended with South Louisiana crude oil, harvested via centrifugation, followed by two saline washes to remove oil. The washed cells were resuspended in saline as 10 fold concentration with glycerol (10% final concentration), and stored at -20 °C until used in the microcosms studies. The cryo enrichment was derived from a sample collected from

131 deep water from the Gulf of Mexico at a depth of 1,240 m and is designed to be
132 representative of a deep water oil degrading consortium. The meso enrichment was
133 derived from a surface water sample from the Gulf of Mexico at a depth of 5 m and is
134 designed to be representative of a surface water oil degrading consortia.
135 In the present study, several frozen stocks of the appropriate enrichment were
136 thawed, combined, and washed once with twice the volume of saline (to remove
137 glycerol). Aliquots of the final resuspended stock (0.5 mL) were then used to inoculate
138 each of the flasks containing GP2 medium amended with ANS oil, dispersant (COREXIT
139 9500), or ANS oil and dispersant at a volumetric dispersant-to-oil ratio of 1:25 (final
140 COREXIT concentration of 1.12 µg/L). Treatments were incubated aerobically by
141 shaking at 200 rpm and incubated at the appropriate temperature (25 °C for the meso and
142 5 °C for the cryo enrichments). Sodium azide killed controls were also prepared to
143 account for abiotic hydrocarbon losses, similarly to the set up described in Campo-
144 Moreno *et al*. (2013) (17). Experiments were conducted in triplicate per treatment with a
145 total of 100 mL of medium in 250 mL flasks. Flasks were sacrificed at 0, 2, 4, 8, 12, 16,
146 24, 32, 40, 48 days. The cryo enrichment had an additional time point at 56 days. At
147 each time point, flasks were sacrificed to measure hydrocarbon residual concentrations
148 and to conduct molecular analyses.
149 Hydrocarbon measurements
150 Subsamples were collected from each flask to determine the concentration of dioctyl
151 sodium sulfosuccinate (DOSS) by liquid chromatography tandem mass spectrometry
152 (LC-MS/MS) and of hydrocarbons by gas chromatography tandem mass spectrometry
153 (GC-MS/MS). The procedures used for DOSS and oil extractions and internal standards

154 used to normalize for DOSS, total alkanes and total aromatics are described elsewhere
155 (17, 19). Hydrocarbons are reported as a ratio to the conservative biomarker hopane,
156 which has been routinely used in determination of biodegradation of oil (21)
157
158 DNA/RNA Extraction and sequencing
159 For each time point, samples (1 mL) were transferred from the microcosms to
160 sterile microcentrifuge tubes. Samples were then centrifuged at 10,000 × g for 10 min.
161 Supernatants were decanted without disturbing cell pellets and tubes were stored at -80
162 °C until nucleic acid extractions. Both RNA and DNA were extracted from the frozen
163 cell pellets with AllPrep DNA/RNA Mini Kit (Qiagen GmbH) (22). RNA extracts were
164 further purified with TURBO DNA-freeTM (Ambion) kits according to the routine
165 DNAse treatment step. Purified RNA was eluted to a final volume of 30 µL and stored at
166 -80°C. cDNA was synthesized from the purified total RNA extracts using the Superscript
167 III (Life Technologies, San Francisco, CA). RNA and DNA concentrations were
168 measured by Qubit RNA High Sensitivity (Life Technologies) and dsDNA High
169 Sensitivity (Life Technologies) assay kits and the Qubit 2.0 Fluorometer (Life
170 Technologies). Aliquots (2 µL) of nucleic acid extracts were used in all quantification
171 reaction mixtures. Concentration of DNA extracts ranged from 0.05 to 1 ng/µL while
172 RNA extracts ranged from 2 to 8 ng/uL, with most within 2 to 5 ng/µL. cDNA synthesis
173 was performed using SuperScript III First-Strand Synthesis kits (Invitrogen) using
174 random hexamer primers according to the manufacturer's instructions. cDNA synthesis
175 was performed the same day of the RNA extraction to maximize RNA and minimize
176 RNA degradation. Aliquots of the RNA extracts were used as PCR templates to verify

177 the absence of DNA contaminants. An 8µL volume of RNA was used for the synthesis
178 generating a final product volume of 21 µL. cDNA was stored at -20 °C and excess RNA
179 was stored at -80 °C.

180 The bacterial composition of each sample was determined via sequencing analysis
181 of the 16S rRNA gene V4 region using the primers 515f/806r as described elsewhere
182 (23). PCR assays used to generate the sequencing libraries were performed in 25 µL
183 volumes using the Ex Taq kit (Takara) with 200 nM each of the forward and reverse
184 primer and 2 µL of template nucleic acid. Cycling conditions involved an initial 5 min
185 denaturing step at 95 °C, followed by 35 cycles at 95 °C for 45 s, 50 °C for 60 s, 72 °C
186 for 90 s, and a final elongation step at 72 °C for 10 min. Each barcode corresponded to
187 an eight-base sequence unique to each sample. Amplicons were visualized on an agarose
188 gel to confirm product sizes. Aliquots of each amplicon were pooled based on band
189 intensity and sequenced on an Illumina Miseq benchtop sequencer using 250 bp paired-
190 end kits at the Cincinnati Children's Hospital DNA Core facility.
191
192 16S rRNA library construction and analysis
193 The resulting DNA sequences were analyzed by using the QIIME version 1.8.0-
194 dev pipeline (24). Paired-end raw reads were assembled with fastq-join (25). The
195 assembled sequences were demultiplexed and quality filtered in QIIME to remove reads
196 with phred scores below 20 (-q 19). Chimera detection was then performed on assembled
197 reads with UCHIME (26, 27). Sequences were then clustered into operation taxonomic
198 units (OTUs, 97% similarity) with UCLUST (26) by using the open reference clustering
199 protocol. The resulting representative sequences were aligned using PyNAST (28) and

given a taxonomic assignment using RDP (29) retrained with the May 2013 Greengenes release. The resulting OTU table was filtered to keep OTUs that were present at greater than 0.005%, and then rarified to the minimum number of remaining sequences in the samples (1670 sequences). Bray-Curtis dissimilarity (30) weighted and unweighted unifrac distances (31) were calculated from the rarefied OTU table with the beta_diversity.py script in QIIME. Several OTUs were originally misclassified as *Methylococcales* spp. Classification of these OTUs via RDP Classifier indicated that the appropriate classification for these OTUs was *Cycloclasticus*. Therefore, we included these OTUs in the *Cycloclasticus* group.

Statistical Analysis of Sequencing Data

In order to test the hypothesis that microbial communities in different treatments were significantly similar to each other and statistically different from other treatments, non-metric multidimensional scaling (NMDS) was used. Weighted Unifrac distances were used to construct two-dimensional NMDS plots. The lowest stress configuration was chosen from 50 iterations of plot construction. Stress values were calculated by the default stress calculation in the NMDS command in the ecodist package. To test if samples from the same treatment were significantly different from each other PERMANOVA (32) analysis was performed on both the Bray Curtis and weighted Unifrac matrices with the Adonis function in the vegan package (33) in R (34). Samples were grouped according to the treatment. PERMANOVA analysis was performed by using 999 permutations. PERMANOVA analysis of all three treatments will indicate if there is a significant difference between all three groups. To distinguish which

223 treatments were different from each other, PERMANOVA analysis was done on subsets
224 of the weighted Unifrac distance matrix that only include samples from two of the three
225 treatments. These pair-wise PERMANOVAs were used to distinguish which treatments
226 were different from each other.

227 The MetagenomeSeq package (35), as implemented in QIIME, was used to
228 identify which OTUs were differentially abundant between treatments for each
229 temperature regime. The MetagenomeSeq package provides a more robust normalization
230 method than total-sum scaling or relative abundance. It uses cumulative sum-scaling, in
231 which counts are divided by the cumulative sum of counts up to a percentile determined
232 using a data-driven approach. Furthermore, MetagenomeSeq employs the zero-inflated
233 Gaussian to account for under sampling of the microbial community in low coverage
234 samples. This enables a more robust analysis of differential abundance between samples
235 or conditions. Each treatment was compared to the other treatments incubated at the
236 same temperature, and OTUs that were significantly different (corrected p value < 0.05)
237 in one treatment compared to the other two were considered to be differentially abundant.

238 **Results and Discussion**

239 Hydrocarbons are rapidly degraded at both 25 and 5 °C

240 Hydrocarbons were measured at various points throughout the course of the experiment.
241 These hydrocarbons were grouped according to total alkanes and PAHs. Many of the
242 hydrocarbon classes were readily degraded with and without COREXIT 9500 (Figure 1),
243 which indicates that the microbial community present in our microcosms contained a
244 robust community of oil-degraders. As Figure 1 depicts, the trend in oil biodegradation
245 in these experiments followed those observed in other studies (10, 36). Total alkane

246 degradation occurred rapidly at 25 °C, with the majority of alkanes being degraded by
247 day 4 in the presence of COREXIT and by day 8 in the absence of COREXIT (Figure
248 1B). In fact, the respective first-order degradation rates were 0.19 ± 0.03 and $0.15 \pm$
249 0.02 d^{-1} in the presence and absence of COREXIT. At 5 °C, minimal alkane
250 degradation was observed the first few days. Substantial alkane degradation was
251 observed after day four and by day 12 the majority of alkanes were degraded both with
252 and without COREXIT. PAHs showed longer lag phases than alkanes at both
253 temperatures (Figure 1C). At 25°C, substantial overall PAH degradation occurred after
254 four days as the extent of removal was 28% in the presence of COREXIT, while in its
255 absence the initial PAHs load remained at near 100%. By day 8, the extent of removal
256 was approximately 77% in both conditions. At 5 °C, PAH degradation was observed to
257 begin after day 12 in the presence of COREXIT 9500 and after day 16 in the absence of
258 COREXIT 9500. Similar trends are observed for particular classes within the broad
259 categories of alkanes and PAHs (Supplemental Figure 1).
260 The effect of COREXIT 9500 was clearly seen when comparing the *n*-alkanes with
261 chain lengths between 30 and 35 (Figure 1D). At 25°C, this group of alkanes was
262 degraded to below detection (< 2 mg/mg hopane) after six days in the presence of
263 COREXIT 9500, while in its absence this group of alkanes was measurable in samples
264 collected at day 16. A similar trend is observed at the lower temperature. In the presence
265 of COREXIT 9500, there is a dramatic decrease in the concentration of this group of
266 alkanes after eight days. However, in the absence of COREXIT 9500 the concentration
267 gradually decreased and reaches the lowest level after 24 days. These results suggest that
268 COREXIT 9500 enhances the rates of oil biodegradation, which is in line with a number

of studies that report increased rates of oil biodegradation in the presence of dispersants (10, 17, 37). This finding is in contrast to the recent paper suggesting that COREXIT 9500 inhibits oil biodegradation (20).

Degradation of DOSS was measured in order to understand the fate of dispersant in these conditions (Figure 1A). At 25 °C, DOSS was quickly degraded in the COREXIT 9500 alone condition, but remained above the detection limit after 48 days. However, in the presence of oil, DOSS was completely degraded after 40 days. This seems to indicate that DOSS degradation occurred more rapidly in the presence of oil at 25 °C. DOSS degradation was much slower at 5 °C with no DOSS degraded over the 56 day experiment. These results are comparable with previous studies that have shown a substantial difference in dispersant degradation at 25 °C and 5°C (17, 38) and persistence of DOSS in the deep ocean (39).

Different treatments select for distinct microbial community composition

Sequencing of the 16S rRNA gene and 16S rRNA gene cDNA transcripts was performed to better understand the effect of COREXIT 9500 on microbial community composition and relative activity of identified members, respectively. In these experiments, there were stark differences between the total community (DNA) and the active community (RNA) composition (Figure 2 and 3). The total microbial community is a measure of all cells present in these samples. Many of these cells may be present at high abundances, but not active during the time of sampling. Therefore, measurement of the microbial community on the RNA level provides insights into which taxa are active during the time of sampling. This approach provides key insights into which of the oil degrading taxa are actively metabolizing the oil components as well as clarifies the

impact of COREXIT addition on the active microbes. Analysis of the total community can often be misleading, as it does not identify the taxa that are actively metabolizing at the time of sampling. While many of the same taxa are present in both the total and the active communities, the dominant taxa are different between the two communities indicating that the key organisms involved in metabolizing hydrocarbons may be incorrectly identified if using only DNA-based total community data.

Multivariate statistics indicate that samples from the same treatment tend to have similar microbial community composition and group together on non-metric multidimensional scaling plots (Figure 3). PERMANOVA analysis indicated that there were statistically significant differences between treatments at both the active and the total community levels (Table 1). These differences indicate that the treatments select for a distinct set of taxa. These differences appear to be the strongest when comparing treatments with and without oil, suggesting that the addition of oil most strongly impacts the community structure. While there are statistically significant differences between the oil-only and oil plus COREXIT treatment, these differences are only weakly significant in some cases (Table 1).

Similarly to what was observed in the Gulf of Mexico during the *Deepwater Horizon* oil spill (DWH), the oil-degrading microbial community at 25°C was dramatically different from the community at 5°C (40) (Figure 2). The 25°C consortium was dominated by unclassified members of the *Vibrio*, *Pseudoidiomarina*, *Marinobacter*, *Alcanivorax*, and *Thallassospira* species. This was similar to the community found in the surface water during the DWH oil spill, which was dominated by members of the *Alteromonadales* and the *Vibrionales* (41). The 5°C consortia were dominated by several

genera of *Flavobacteria*, as well as *Oceanospirillales* related to *Alcanivorax* and *Oleispira*. Additionally, *Colwellia* spp. were found to be present in the majority of samples during the incubation. Microbes classified as *Cycloclasticus* were present in the later time points of many conditions at both 25°C and 5°C. These same taxa were present at high abundance in the deep-water plume during the DWH oil spill (40). Therefore, the major oil-degrading taxa in these enriched consortia are related the dominant members of the microbial community that responded to the spilled oil in the Gulf of Mexico (42).

Treatment effects on the active microbial community at 25 °C

Analysis of the composition of the active bacterial community provides a clearer understanding of the microbial taxa actively responding to the treatment. A number of taxa were present at different relative abundances in the active community compared to the total community. For example, the total community at 25 °C was dominated *Vibrionaceae* (up to 80% of the recovered reads), whereas the *Vibrionaceae* only represented ~25% of recovered reads in the active community. Members of the *Thallasospira*, *Marinobacter*, and *Pseudoidiomarina* were present at much higher relative abundance in the active community compared to the total community. Additionally, *Marinobacter* represented between 2 – 7% of the total community across all treatments. In the active community, *Marinobacter* represented 1.7% of recovered reads in the COREXIT 9500-only treatment. However, in oil-amended conditions *Marinobacter* comprised 27 and 17% of reads in the oil-only and the oil plus COREXIT 9500 treatments, respectively. This finding suggests that *Marinobacter* is stimulated by the addition of oil. *Marinobacter* are a well-known oil-degrading bacteria (43) that were present in the Gulf of Mexico during the DWH oil spill (44).

The role of *Vibrionales* in these consortia is somewhat enigmatic. *Vibrio* spp. have been identified as responding to the DWH oil spill in beach sediments (45, 46); however, evidence for degradation of hydrocarbons by *Vibrio* is sparse. There is some previous evidence of their ability to use hydrocarbons (47). One study performed during the DWH oil spill demonstrated an enrichment of *V. vulnificus* in tar balls collected from Gulf of Mexico beaches (48). Conversely, a number of *V. parahaemolyticus* isolates from water and sediments from the Gulf of Mexico were shown to not be able to degrade PAHs (49). Further, little to no oil consumption was observed for *Vibrio* strains isolated from Gulf of Mexico beach sands (46). *Vibrio* are common inhabitants of the ocean and are known to grow on a wide range of organic compounds (47). It is therefore possible that these *Vibrio* species are responding to the spilled oil as well as metabolizing the compounds produced by other oil-degrading taxa.

Treatment effects on the active microbial community at 5 °C

Differences between the active and total community were also observed at 5 °C. The *Flavobacteriacea* related to *Winogradskyella* were more dominant members of the active community in the COREXIT 9500 only condition compared to the oil-amended conditions. *Oleispira* were also present at high abundance in the total community in all conditions (on average between 14 – 17% of recovered reads). *Oleispira* represented a much higher proportion of the active community in the oil-amended conditions (on average 15% of the oil-only and 9.4% of oil plus COREXIT 9500) compared to the total community, but were only a minor constituent of the active community in COREXIT 9500-only conditions (on average 2.9%). The increase in the proportion of *Oleispira* in the oil-amended conditions suggests that *Oleispira* is stimulated by the addition of oil in

these microcosms. *Oleispira* spp. are known as psychrophilic alkane-degraders that were originally described as an isolate from the Antarctic (50). The closest cultured representative to the *Oceanospirillales*, which dominated the deep-water plume at the early stages of the *Deepwater Horizon* oil spill, is *O. antarctica* (51).

Cycloclasticus spp. have been shown to be numerically dominant members of the *in situ* microbial community that responded to the *Deepwater Horizon* oil spill (40, 44). Many of the described species of *Cycloclasticus* are able to degrade a variety of PAHs. In our microcosms, sequences classified as *Cycloclasticus* were recovered from the active communities at both temperatures. At 5 °C, *Cycloclasticus* spp. were present at low abundance in the early time points (on average 0.8% at Day 8) and remained at low abundance until day 24 in the only oil condition and Day 16 in the oil plus COREXIT 9500 condition, when they increased to 5% of the active community. This increase in the relative abundance of *Cycloclasticus* corresponds with the beginning of PAH degradation at 5°C (Figure 1C), further supporting the role of *Cycloclasticus* spp. in degrading PAHs in these microcosms.

Colwellia spp. were also important members of the deep water plume at later time points in the spill (40, 52). *Colwellia* are ubiquitous psychrophilic heterotrophs. Data from experiments performed during the spill indicated that they degraded gaseous hydrocarbons, namely propane, ethane, and benzene (40). *Colwellia* were on average 4.9% of the total community in all conditions, whereas in the active community, they were 13.2% in the COREXIT 9500-only condition, and only 2.1 and 3.8% of the active community in oil-only and oil plus COREXIT 9500 treatments, respectively. In the COREXIT 9500-only condition, reads classified as *Colwellia* increased over the course

384 of the experiment; increasing from 0.9% to a maximum of 22.4% on day 24. This
385 increase in *Colwellia* in the COREXIT only conditions may be due to metabolism of
386 COREXIT components by *Colwellia* as suggested previously (15, 41).
387 Analysis of these microcosms further confirms that the oil-degrading microbial
388 communities derived from surface water (meso enrichment) are distinct from the
389 microbial communities derived from deep water (cryo enrichment). The surface
390 microbial community was able to rapidly degrade the majority of the components of oil
391 and COREXIT 9500. The surface microbial community was dominated by well
392 characterized oil-degrading microbes such as *Marinobacter* species, which are common
393 mesophilic oil-degrader. The deep-water community was composed of distinct taxa
394 compared to the surface community and degraded oil at a different rate than under
395 surface water conditions. These differences are in part due to differences in temperature.
396 While it is expected that low temperature would act to slow the rates of oil
397 degradation, we find that the deep-water community is dominated by many of cold-
398 adapted oil-degrading taxa. This suggests that deep water oil biodegradation would be
399 more rapid than would be expected based on the Q10 approach (53). Many of the same
400 cold-adapted oil-degrading taxa found in the 5°C microcosms were found to be important
401 in responding to oil spills in other cold locations (50, 54, 55). The presence of many
402 these same taxa in other oil-impacted locations suggests that there is a ubiquitous
403 complement of cold-adapted oil degraders that may respond to oil spills in cold climates.
404 Interestingly, one of the dominant oil-degraders in the 5 °C incubation was an
405 *Alcanivorax* sp. *Alcanivorax* are ubiquitous oil-degraders commonly found in surface
406 seawater. They were not reported as being dominant in deep water plume during the

DWH oil spill. Previous work has demonstrated that *Alcanivorax* sp. were dominant in oil-impacted seawater when sufficient nutrients are present. It is possible that in the deep ocean, nutrient limitation impedes the growth of *Alcanivorax* species. However, in these microcosms, nutrients are supplied in the artificial seawater allowing for the proliferation of these *Alcanivorax* strains. While *Alcanivorax* species have been isolated from the deep sea, they are not typically thought of as cold-adapted microbes. Therefore, the fact that *Alcanivorax* species dominate the active community at 5 °C suggests the potential for cold-adapted members of the *Alcanivorax* to be important responders in cold environments assuming that sufficient nutrients are present.

Microbial communities are distinct in different locations and may respond differently due to local conditions. Many of the oil degraders present in this model community are representative of microbes shown to respond to oil spills throughout the world (2). While this model community may not provide information about how a specific location would respond to an oil spill, it does provide a system in which we can reproducibly study the impact of various response strategies on a representative oil degrading consortia.

Our results indicate distinct microbial community dynamics in response to oil as well as other treatments. Microbial communities exposed to oil demonstrate a coordinated dynamic with the initial responders typically being alkane degraders followed by microbes specialized in degrading more recalcitrant compounds such as aromatic compounds. Previous studies have indicated that environmental microbial communities exhibit dynamics to degrade the preferred carbon source (42). The oil degrading microbial communities in these enrichments exhibit similar dynamics with the

initial bloom of alkane degraders (*Marinobacter*, *Alcanivorax*, etc.) followed by an increase in known aromatic hydrocarbon degraders (*Cycloclasticus*, *Alteromonadales*, *Thallasospira*, etc.). Growth on the decaying bloom of oil degrading bacteria was observed during the *DWH* spill (42), but was not observed in these enrichments..

Differential effects of COREXIT 9500 on community composition based on temperature

The primary goal of this study was to assess the effect of COREXIT 9500 on the oil biodegradation and microbial community structure. Since the active community should be the most diagnostic for the effect of COREXIT 9500, we will only discuss the differences in the active community between treatments. PERMANOVA analysis indicated that there were significant differences in the microbial community structure between treatments at both temperatures. Pairwise PERMANOVAs were used to determine which treatments were significantly different. At 25 °C, there was a significant difference in the microbial community present in all three treatments (Table 1). However, at 5 °C, there were significant differences between the COREXIT 9500-only conditions and the oil-containing conditions (oil alone and oil plus COREXIT 9500). There was no statistically significant difference between the oil-only and the oil plus COREXIT 9500 treatments at 5 °C (PERMANOVA p-value: 0.367). These findings suggest that at 5°C, the addition of COREXIT 9500 to oil-amended microcosms has a negligible impact on the community structure compared to the structure in the oil-only conditions. This would suggest that at cold temperatures the active community structure is minimally impacted by the addition of COREXIT 9500. The observation that COREXIT addition stimulated oil biodegradation at 5 °C despite the minimal impact on

453 the microbial community, may imply that the COREXIT increases the bioavailability of
454 oil for the natural oil degrading population. Alternate explanations for the impact of
455 COREXIT on community structure could be due to COREXIT serving as a carbon source
456 for microbes in these consortia. At 5°C there is little DOSS degradation. However, there
457 was substantial DOSS degradation at 25°C. This difference in DOSS degradation could
458 indicate that the distinct community structure in response to COREXIT could be due to
459 microbes consuming the COREXIT components.

460 COREXIT 9500 differentially impacts members of the same taxonomic grouping

461 To better understand which taxa were most affected by COREXIT 9500 at 25 °C,
462 differentially abundant taxa were identified between the only oil and the oil plus
463 COREXIT 9500 conditions. Forty operational taxonomic units (OTUs) were identified
464 as being differentially abundant in the oil plus COREXIT 9500 condition compared to the
465 oil-only condition at 25 °C (Table 2). No OTUs were identified as showing significantly
466 different abundances between the oil-only and oil plus COREXIT 9500 treatment at 5 °C,
467 which further supports the limited effect of COREXIT 9500 on these microbial consortia
468 at 5 °C. The differentially abundant OTUs were classified into eight orders representing
469 10 families. Of the 40 differentially abundant OTUs, 17 were more abundant in the oil
470 and COREXIT 9500 condition, whereas 23 were more abundant in the oil-only condition.
471 Five of the differentially abundant OTUs were assigned as an unclassified
472 *Gammaproteobacteria*. Three of the unclassified *Gammaproteobacterial* OTUs were
473 enriched in the oil plus COREXIT 9500 condition, whereas two of them were enriched in
474 the oil-only condition.

A previous study demonstrated that COREXIT 9500 addition resulted in decreased viability of a *M. hydrocarbonoclasticus* isolate obtained from a Gulf of Mexico beach (14). In the present study, *Marinobacter* comprised a large proportion of the active community in the oil-amended conditions incubated at 25 °C. *Marinobacter* spp. were present at an average relative abundance of 27% in the oil-only conditions and 17% in the oil plus COREXIT 9500 condition. This result could be interpreted as an inhibitory effect of COREXIT 9500 on *Marinobacter* species, however, changes in relative abundance could be due to a variety of factors. One possible explanation for this change in relative abundance is that there were less *Marinobacter* cells in the oil plus COREXIT 9500 condition. Alternatively, since these data were obtained from cDNA libraries, it could be that *Marinobacter* were present, yet less active in the oil plus COREXIT 9500 conditions. A third possible explanation is that *Marinobacter* spp. remain at the same level (either cells or activity) in both treatments, but other organisms increased in abundance or activity in the oil plus COREXIT 9500 condition. This third scenario would result in an apparent decrease in the relative abundance of *Marinobacter*. Further complicating matters is the fact that the relative abundance measured was the combined abundance of all of the OTUs classified as *Marinobacter*. Therefore, an examination of difference of the relative abundance of specific OTUs would provide a more accurate estimation of the effect of COREXIT 9500 between treatments.

Eighteen *Marinobacter* OTUs were identified as being differentially abundant between treatments. Eight of these *Marinobacter* OTUs showed enrichment in the oil plus COREXIT 9500 condition, and the other ten were enriched in the oil-only condition relative to the oil plus COREXIT 9500 condition. This would indicate that, while there

498 were certain *Marinobacter* OTUs that may be sensitive to the addition of COREXIT
499 9500, many of the *Marinobacter* OTUs were stimulated by the addition of COREXIT
500 9500. For example, one OTU was on average present at 0.3% and 0.4% in the COREXIT
501 9500 only and the oil-only samples, respectively. However, in the presence of both oil
502 and COREXIT 9500 this OTU increased to 2.2%, suggesting that this specific
503 *Marinobacter* population was stimulated by the addition of COREXIT 9500 when oil is
504 present. This potential stimulation would be in contrast to a previous report, which found
505 inhibition of an *M. hydrocarbonclasticus* isolate with the addition of COREXIT 9500
506 (14). This discrepancy could be due to the high-levels of COREXIT 9500 used in the *M.*
507 *hydrocarbonoclasticus* study. In this present study, the concentration of COREXIT 9500
508 was 1.12 µg/L and may not be inhibitory to all *Marinobacter* species.
509 There were some examples of *Marinobacter* OTUs that exhibited the opposite
510 trend. For example, one OTU was present at 0.7% of cDNA libraries with COREXIT
511 9500 alone. In the samples with oil, this OTU increased to 21% of the recovered
512 sequences. When oil and COREXIT 9500 are present, the abundance of this OTU was
513 only 10% of the library. Thus, there are fewer sequences recovered in the oil and
514 COREXIT 9500 treatment compared to the oil alone. This two-fold decrease in
515 recovered sequences could be indicative of an inhibitory effect of COREXIT 9500
516 addition on this OTU. Similar phenomena were observed with many of the other
517 differentially abundant OTUs, with some members of the same genus showing higher
518 abundance in the oil-only versus oil and COREXIT 9500 condition and visa versa.
519 However, there were some differentially abundant taxa that were only enriched in one
520 condition. For example, three OTUs, classified as *Alcanivorax* spp., were enriched in the

521 only oil condition compared to the oil plus COREXIT 9500 condition. One of the
522 differentially abundant *Alcanivorax* OTUs was the dominant *Alcanivorax* OTU found in
523 these microcosms. At 25°C, this OTU comprised on average 6.7% of the oil-only cDNA
524 communities and 3.5% of the oil plus COREXIT 9500 communities. This statistically
525 significant difference suggests that in the COREXIT 9500-amended microcosms, this
526 *Alcanivorax* sp. was not as competitive. Interestingly, in the 5°C incubation, this same
527 OTU comprised on average 56.8% and 59.5% of the active community in the oil-only
528 and the oil plus COREXIT 9500 microcosms, respectively. This suggests that at 25°C
529 there is a possibility that this OTU is inhibited by COREXIT 9500; in contrast, at 5°C the
530 addition of COREXIT 9500 has no effect. Therefore, the effect of COREXIT 9500
531 inhibition may be species-specific and dependent upon temperature. This is in agreement
532 with recent studies that demonstrated that COREXIT 9500 did not affect the growth of *A.*
533 *borkumensis* (Liu and Hazen, unpublished results).
534
535 **Conclusions**
536 The goal of this study was to provide additional insights into the effects of COREXIT
537 9500 on biodegradation of ANS crude oil as well its effects on the community structure
538 of oil-degrading microbes. These results combine to show that biodegradation is
539 enhanced by the addition of COREXIT 9500. Our results indicate that microbial
540 community structure is only impacted by COREXIT 9500 addition at 25 °C and not at 5
541 °C. The effect of COREXIT 9500 addition at 25 °C is stronger in the active community.
542 We have demonstrated that while there were some OTUs negatively impacted by the
543 addition of COREXIT 9500, a similar number of differentially abundant OTUs were

stimulated by the addition of COREXIT 9500. Our results suggest that the effect of COREXIT 9500 is somewhat species specific and even within a species, there can be differing effects depending on the individual strain/organism. Multiple species have the ability to degrade single classes of hydrocarbons. Therefore, if one species is inhibited by COREXIT 9500 addition, another may grow in its place. This study confirms previous studies that have shown a positive impact of dispersants on rates of oil biodegradation. While these results demonstrate that the microbial community is impacted by COREXIT addition, it appears to be a species-specific phenomenon.

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Figure Legends

Figure 1: Oil Biodegradation. (A) Degradation of DOSS in the presence and absence of ANS oil. The left side of panel A shows degradation of DOSS in the presence of ANS. The right side of panel A shows DOSS degradation in the absence of ANS. (B-D) Comparison of biodegradation of ANS crude oil in the presence (left) and absence (right) of COREXIT 9500 at 25°C (open symbols) and 5°C (filled symbols). (B) Biodegradation of hopane-normalized total alkanes. (C) biodegradation of hopane-normalized total PAHs (D) biodegradation of *n*-alkanes (nC30 – nC35).

Figure 2 Microbial Community Dynamics. Analysis of the microbial community structure at the total (DNA – Left) and active (cDNA- right) levels for the 25°C (top) and 5°C (bottom) microcosms. Taxa are shown grouped at the genus level. Sequences from replicate microcosms were pooled and shown as a single bar for each time point and condition.

Figure 3: Comparison of microbial community structure. Non-metric multidimensional scaling of weighted unifrac distances. Samples with only COREXIT 9500 were colored in gray, samples with oil-only were colored in orange, and samples with oil plus COREXIT 9500 were colored in blue. Comparison of the total microbial community composition in the 5°C microcosm (A) and the 25°C microcosm (C). The microbial community composition was compared in the active community of the 5°C microcosm (B) and the 25°C microcosm (D) Stress for the configuration of the different plots are 0.0622 (A), 0.0681 (B), 0.10604 (C), and 0.1997 (D).

Table 1: Pairwise PERMANOVAs of weighted unifrac distances

Temp.	Nucl. Acid	Comparison	pvalue	F stat	R2
5°C	DNA	COREXIT v. Oil+COREXIT	0.001	18.78	0.2446
		Oil v. Oil+COREXIT	0.012	3.9657	0.06613
		COREXIT v Oil	0.001	44.231	0.43266
	cDNA	COREXIT v. Oil+COREXIT	0.001	78.728	0.56344
		Oil v. Oil+COREXIT	0.367	1.0117	0.01714
		COREXIT v Oil	0.001	89.818	0.59554
25°C	DNA	COREXIT v. Oil+COREXIT	0.004	4.1372	0.06996
		Oil v. Oil+COREXIT	0.047	2.6213	0.04799
		COREXIT v Oil	0.001	9.684	0.14971
	cDNA	COREXIT v. Oil+COREXIT	0.001	6.5336	0.10691
		Oil v. Oil+COREXIT	0.001	11.935	0.18668
		COREXIT v Oil	0.001	20.136	0.26799

Table 2: Differentially abundant taxa between samples.

Family	Genus	Number of OTUs enriched in oil-only	Number of OTUs enriched in oil plus COREXIT 9500
Nocardiaceae	*Rhodococcus*	0	1
Phycisphaerales	Unclassified	0	1
Rhodobacterales	*Hyphomondadaceae*	2	0
Rhodobacterales	*Rhodobacteraceae*	3	0
Sphingomonadlaes	*Erythrobacteraceae*	0	1
Gammaproteobacteria	Unclassified	2	3
Alteromonadaceae	*Marinobacter*	10	8
Idiomarinaceae	*Pseudidiomarina*	1	1
Alcanovoraceae	*Alcanovorax*	0	3
Vibrionaceae	Unclassified	3	1
	Total	21	19

Figure 1

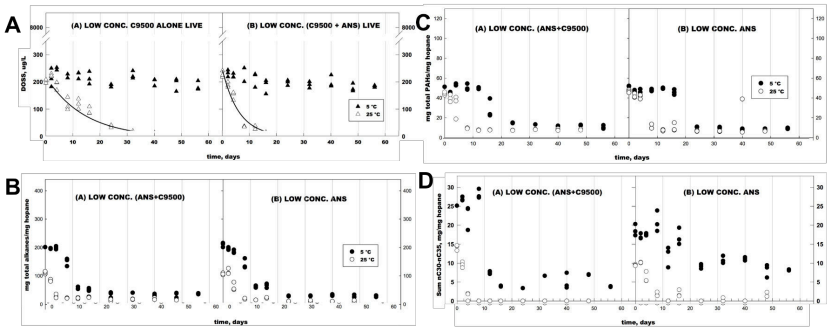

Figure 2

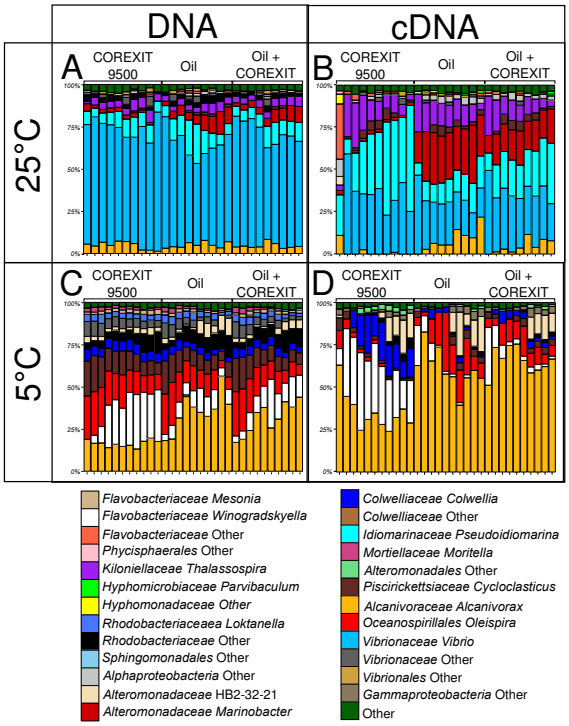

Figure 3