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Corexit 9500 Enhances Oil Biodegradation and Changes Active Bacterial Community Structure of Oil-Enriched Microcosms

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ABSTRACT

To better understand the impacts 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 using both DNA and RNA extracts as the 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). Slight increases in biodegradation were 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 were dominated by *Vibrio*, *Idiomarina*, *Marinobacter*, *Alcanivorax*, and *Thalassospira* species, while the 5°C consortia were dominated by several species of the genera *Flavobacterium*, *Alcanivorax*, and *Oleispira*. Most of 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 bacterial community structure of the 5°C consortia, while at 25°C, a decrease in the relative abundance of *Marinobacter* was observed. At 25°C, *Thalassospira*, *Marinobacter*, and *Idiomarina* were present at higher relative abundances 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 dispersants such as Corexit 9500 can be used to treat oil spills, 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.

INTRODUCTION

Diverse marine microbes have the ability to degrade crude oil under both aerobic and anaerobic conditions (**1**, **2**). Crude oil is a complex mixture of hydrocarbons, with constituents including both branched and straight chain alkanes as well as polycyclic aromatic hydrocarbons (PAHs). In addition to the natural inputs of oil into the marine environment (**3**), oil has been released via accidental oil spills. These oil spills have the potential to drastically impact ecosystems and result in long-term environmental changes (**4**, **5**). Various strategies have been employed to aid in the cleanup of oil spills, including dispersant application (**6**). Chemical dispersants contain a mixture of chemicals designed to break up an oil slick by reducing the interfacial surface tension of the oil, causing it to form micron-sized droplets. These droplets can then be diluted in the water column preventing the formation of a surface slick. Indigenous oil-degrading microbes are believed to degrade these dispersed oil droplets, with smaller droplets being more rapidly degraded (**7**). During the *Deepwater Horizon* oil spill, 2.1 million gallons of the chemical dispersant Corexit 9500 were applied at both the surface and at the seafloor (**8**). There is debate about the effect of Corexit 9500 on the rates of oil biodegradation as well as its effects on the microbial community composition. Several reports from diverse marine locations have demonstrated that the addition of Corexit 9500 enhances oil biodegradation (**7**, **9–12**). Other studies have shown a neutral or inhibitory effect of Corexit 9500 addition on microbial activity (**13–15**). A number of factors could explain these discrepancies, including differences in the experimental design, the lengths of the experiments, the water sources (including handling and storage), and the 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**). The results from that study 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. is underscored by the very high Corexit 9500 concentration used in their study (**9**). 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. (**9**) used a dispersant-to-oil ratio of 1:1.6. Another recent study investigated the impact of Corexit 9500 on both the biodegradation of the water accessible fraction (WAF) of oil and the microbial community

structure ([15](#)). The authors concluded 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 the focus of oil biodegradation studies. Corexit 9500 has been shown to effectively disperse ANS crude oil in wave tank experiments ([17](#)) as well as to enhance the 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 the biodegradation of ANS crude oil and the impact of Corexit addition on microbial community structures of two hydrocarbon-degrading consortia. Next-generation sequencing of the 16S rRNA gene using both DNA and RNA extracts as the sequencing targets was used to study the microbial community composition. By comparing the results at both the DNA and RNA levels, we determined the effect of Corexit 9500 on the total bacterial community structure (DNA) as well as on the active bacterial fraction (RNA). The goal of this study was to 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.

RESULTS AND DISCUSSION

Hydrocarbons are rapidly degraded at both 25 and 5°C. Hydrocarbons were measured at various points throughout the course of the experiment. These hydrocarbons were grouped according to total alkanes and PAHs. Many of the hydrocarbon classes were readily degraded with and without Corexit 9500 (**[Fig. 1](#)**), which indicates that the microbial community present in our microcosms contained a robust community of oil degraders. As **[Fig. 1](#)** depicts, the trend in oil biodegradation in these experiments followed those observed in other studies ([10](#), [18](#)). Total alkane degradation occurred rapidly at 25°C, with the majority of alkanes being degraded by day 4 in the presence of Corexit and by day 8 in the absence of Corexit (**[Fig. 1B](#)**). In fact, the respective first-order degradation rates were 0.19 ± 0.03 and 0.15 ± 0.02 day^{-1} in the presence and absence of Corexit. At 5°C, minimal alkane degradation was observed during the first few days. Substantial alkane degradation was observed after day 4, and by day 12, the majority of alkanes were degraded with and without Corexit. PAHs showed longer lag phases than alkanes at both temperatures (**[Fig. 1C](#)**). At 25°C, substantial overall PAH degradation occurred after 4 days as the extent of removal was 28% in the presence of Corexit, while in its absence, the initial PAH load remained at near 100%. By day 8, the extent of removal was approximately 77% under both conditions. At 5°C, PAH degradation was observed to begin after day 12 in the presence of Corexit 9500 and after day 16 in the absence of Corexit 9500. Similar trends were observed for particular classes within the broad categories of alkanes and PAHs (see Fig. S1 in the supplemental material).

FIG 1

Oil biodegradation. (A) Degradation of DOSS in the absence (left) and presence (right) of ANS oil. (B to D) Comparison of biodegradation of ANS crude oil in the presence (left) and absence (right) of Corexit 9500 at 25°C (open symbols) and at 5°C (filled symbols). (B) Biodegradation of hopane-normalized total alkanes. (C) Biodegradation of hopane-normalized total PAHs. (D) Biodegradation of *n*-alkanes (nC30 to nC35).

The effect of Corexit 9500 was clearly seen when comparing the *n*-alkanes with chain lengths of between 30 and 35 (**Fig. 1D**). At 25°C, this group of alkanes was degraded to below detection (<2 mg/mg hopane) after 6 days in the presence of Corexit 9500, while in its absence, this group of alkanes was measurable in samples collected at day 16. A similar trend was observed at the lower temperature. In the presence of Corexit 9500, there was a dramatic decrease in the concentration of this group of alkanes after 8 days. However, in the absence of Corexit 9500, the concentration gradually decreased and reached the lowest level after 24 days. These results suggest that 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**, **19**, **20**). This finding is in contrast to a recent paper suggesting that Corexit 9500 inhibits oil biodegradation (**21**).

The degradation of dioctyl sodium sulfosuccinate (DOSS) was measured to understand the fate of the dispersant under these conditions (**Fig. 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 those from previous studies that have shown a substantial difference in dispersant degradation at 25°C and 5°C (**19**, **22**) and the persistence of DOSS in the deep ocean (**23**).

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 effects of Corexit 9500 on microbial community composition and on the relative activity of identified members, respectively. In these experiments, there were stark differences between the total community (DNA) and the active community (RNA) compositions (**Fig. 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 at the time of sampling. Therefore, the measurement of the microbial community at the RNA level provides insights into which taxa are active at 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 clarifying the impact of Corexit addition on the active microbes. An 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.
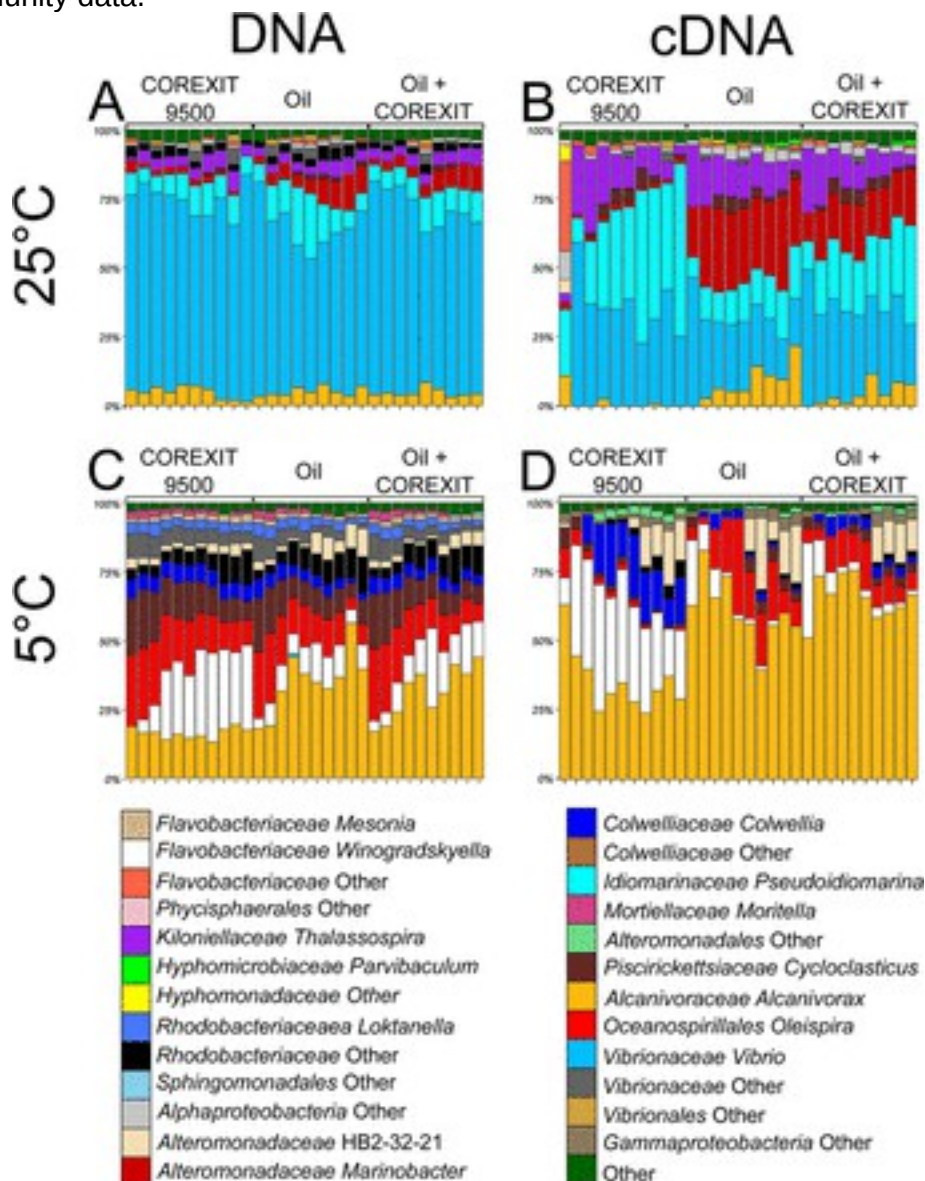
FIG 2

Microbial community dynamics. Analyses of the microbial community structures at the total (A, C) and active (B, D) cDNA 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.
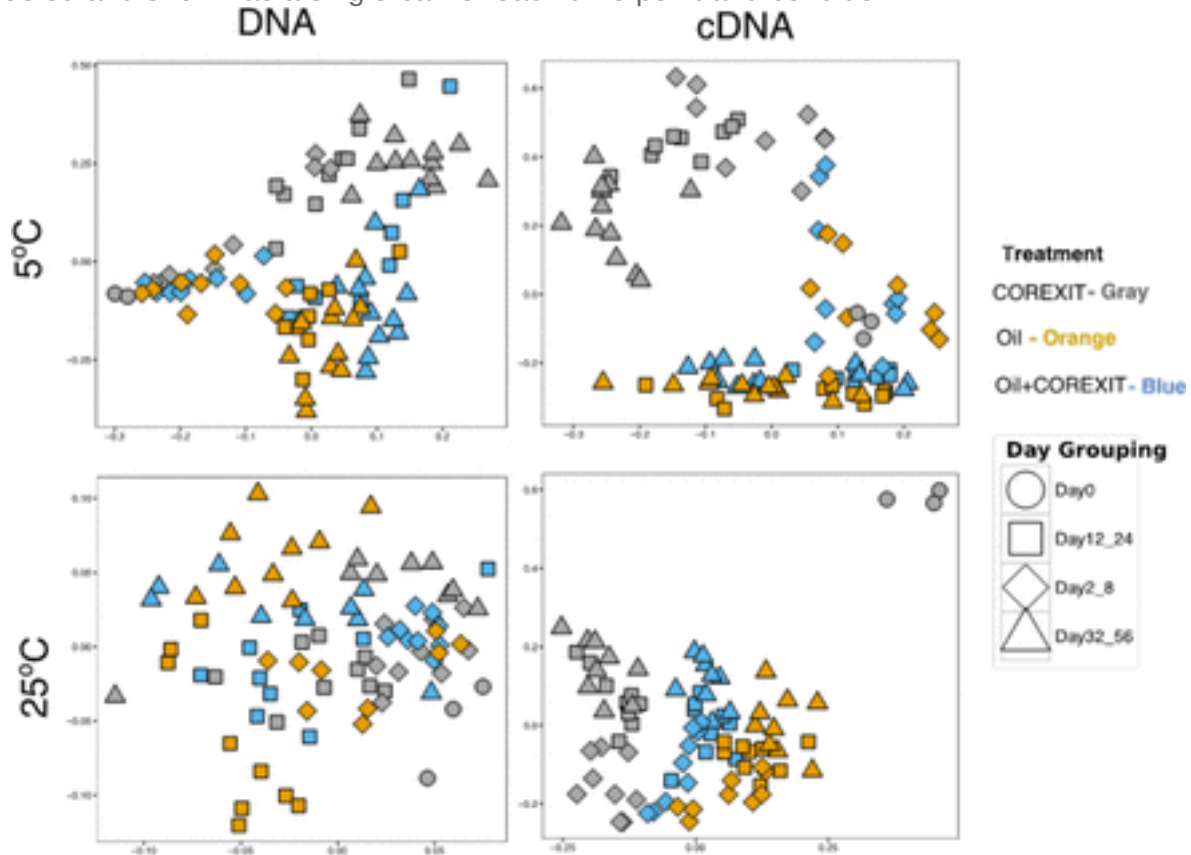
FIG 3

Comparison of microbial community structure. Nonmetric multidimensional scaling of weighted UniFrac distances. Samples with only Corexit 9500 are colored gray, samples with oil-only are colored orange, and samples with oil plus Corexit 9500 are colored blue. Comparison of the total microbial community compositions in the 5°C (top left) and 25°C (bottom left) microcosms. The microbial community compositions were compared in the active communities of the 5°C (top right) and 25°C (bottom right) microcosms. Stresses for the configuration of the different plots are 0.0622 (top left), 0.0681 (top right), 0.10604 (bottom left), and 0.1997 (bottom right).

Multivariate statistics indicate that samples from the same treatment tend to have similar microbial community compositions and group together on nonmetric multidimensional scaling plots (**Fig. 3**). Permutational multivariate analysis of variance (PERMANOVA) 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 distinct sets 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 treatments, these differences are only weakly significant in some cases (**Table 1**).

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- **View popup**

TABLE 1

Pairwise PERMANOVAs of weighted UniFrac distances

Similar to what was observed in the Gulf of Mexico during the *Deepwater Horizon* (DWH) oil spill, the oil-degrading microbial community at 25°C was dramatically different from the community at 5°C (**24**) (**Fig. 2**). The 25°C consortium was dominated by unclassified members of the *Vibrio*, *Idiomarina*, *Marinobacter*, *Alcanivorax*, and *Thalassospira* genera. This was similar to the community found in the surface water during the DWH oil spill, which was dominated by members of *Alteromonadales* and *Vibrionales* orders (**25**). The 5°C consortia were dominated by several genera of *Flavobacteriaceae*, as well as by *Oceanospirillales*related to *Alcanivorax* and *Oleispira*. Additionally, *Colwellia* spp. were found to be present in the majority of samples during the incubation. Bacteria 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 a high abundance in the deep-water plume during the DWH oil spill (**24**). Therefore, the major oil-degrading taxa in these enriched consortia are related to the dominant members of the microbial community that responded to the spilled oil in the Gulf of Mexico (**26**).

Treatment effects on the active microbial community at 25°C. An 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 with that of the total community. For example, the total community at 25°C was dominated by *Vibrionaceae* (up to 80% of the recovered reads), whereas *Vibrionaceae* only represented ~25% of recovered reads in the active community. Members of *Thalassospira*, *Marinobacter*, and *Idiomarina* genera were present at much higher relative abundances in the active community compared with those in the total community.

Additionally, *Marinobacter* represented between 2 to 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, under oil-amended conditions, *Marinobacter* comprised 27 and 17% of reads in the oil-only and oil-plus-Corexit 9500 treatments, respectively. This finding suggests that *Marinobacter* spp. are stimulated by the addition of oil. *Marinobacter* spp. are well-known oil-degrading bacteria (**27**) that were present in the Gulf of Mexico during the DWH oil spill (**28**).

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 (**29**, **30**); however, evidence for the degradation of hydrocarbons by *Vibrio* is sparse. There is some previous evidence of its ability to use hydrocarbons (**31**). One study performed during the DWH oil spill demonstrated an enrichment of *V. vulnificus* in tar balls collected from Gulf of Mexico beaches (**32**). 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 (**33**).

Furthermore, little-to-no oil consumption was observed for *Vibrio* strains isolated from Gulf of Mexico beach sands (**30**). *Vibrio* is a common inhabitant of the ocean and is known to grow on a wide range of organic compounds (**31**). Therefore, it is 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 communities were also observed at 5°C.

The *Flavobacteriaceae* related to *Winogradskyella* were more dominant members of the active community under the Corexit 9500-only condition than under the oil-amended conditions. *Oleispira* was also present at a high abundance in the total community under all conditions (on average, between 14 to 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) than of the total community but was 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 from an isolate from the Antarctic (**34**). 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* (**35**).

Cycloclasticus spp. have been shown to be numerically dominant members of the *in situ* microbial community that responded to the *Deepwater Horizon* oil spill (**24**, **28**). 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 a low abundance in the early time points (on average, 0.8% at day 8) and remained at a low abundance until day 24 under the oil-only condition and day 16 under 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 (**Fig. 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 (**24**, **36**). *Colwellia* is a ubiquitous psychrophilic heterotroph. Data from experiments performed during the spill indicated that it degraded gaseous hydrocarbons, namely, propane, ethane, and benzene (**24**). *Colwellia* comprised, on average, 4.9% of the total community under all conditions, whereas in the active community, it comprised 13.2% under 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. Under the Corexit 9500-only condition, reads classified as *Colwellia* increased over the course of the experiment, increasing from 0.9% to a maximum of 22.4% on day 24. This increase in *Colwellia* under the Corexit-only conditions may be due to the metabolism of Corexit components by *Colwellia* as suggested previously (**15**, **25**).

Analysis of these microcosms further confirms that the oil-degrading microbial communities derived from surface water (meso enrichment) are distinct from the microbial communities derived from deep water (cryo enrichment). The surface microbial community was able to rapidly degrade the majority of the components of oil and Corexit 9500. The surface microbial community was dominated by well-characterized oil-degrading microbes such as *Marinobacter* species, which are common mesophilic oil degraders. The deep-water

community was composed of distinct taxa compared with those of the surface community and degraded oil at a different rate than under surface water conditions. These differences are partly due to the differences in temperatures.

While it is expected that low temperatures would act to slow oil degradation, we found that the deep-water community is dominated by many cold-adapted oil-degrading taxa. This suggests that deep-water oil biodegradation would be more rapid than would be expected based on the Q10 approach (**37**). Many of the same cold-adapted oil-degrading taxa found in the 5°C microcosms were found to be important in responding to oil spills in other cold locations (**34**, **38**, **39**). The presence of many of these same taxa in other oil-impacted locations suggests that there is a ubiquitous complement of cold-adapted oil degraders that may respond to oil spills in cold climates. Interestingly, one of the dominant oil-degraders in the 5°C incubation was an *Alcanivorax* sp. *Alcanivorax* is a ubiquitous oil degrader commonly found in surface seawater. It was not reported as being dominant in deep-water plumes during the DWH oil spill. Previous work has demonstrated that *Alcanivorax* sp. were dominant in oil-impacted seawater when sufficient nutrients were 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* genus 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 microbes in 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 consortium. 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 (**26**). 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*, *Thalassospira*, etc.). Growth on the decaying bloom of oil-degrading bacteria was observed during the DWH spill (**26**), 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 effects of Corexit 9500 on oil biodegradation and the 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 communities between treatments. A PERMANOVA indicated that there were significant differences in the microbial community structures 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

communities 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, $P = 0.367$). 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 with the structure in the oil-only condition. 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 the microbial community may imply that Corexit increases the bioavailability of oil for the natural oil-degrading population. An alternate explanation for the impact of Corexit on community structure might be that Corexit serves as a carbon source for microbes in these consortia. At 5°C there was little DOSS degradation. However, there was substantial DOSS degradation at 25°C. This difference in DOSS degradation may indicate that the distinct community structure in response to Corexit might be due to microbes consuming the Corexit components.

Corexit 9500 differentially impacts members of the same taxonomic grouping. To better understand which taxa were most affected by Corexit 9500 at 25°C, differentially abundant taxa were identified between the oil-only and oil-plus-Corexit 9500 conditions. Forty operational taxonomic units (OTUs) were identified as being differentially abundant under the oil-plus-Corexit 9500 condition compared with those under the oil-only condition at 25°C (**Table 2**). No OTUs were identified as showing significantly different abundances between the oil-only and oil-plus-Corexit 9500 treatment at 5°C, which further supports the limited effect of Corexit 9500 on these microbial consortia at 5°C. The differentially abundant OTUs were classified into eight orders representing 10 families. Of the 40 differentially abundant OTUs, 17 were more abundant under the oil and Corexit 9500 condition, whereas 23 were more abundant under the oil-only condition. Five of the differentially abundant OTUs were assigned to unclassified *Gammaproteobacteria*. Three of the unclassified gammaproteobacterial OTUs were enriched under the oil-plus-Corexit 9500 condition, whereas two of them were enriched under the oil-only condition.

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TABLE 2

Differentially abundant taxa between samples

A previous study demonstrated that Corexit 9500 addition resulted in a decreased viability of an *M. hydrocarbonoclasticus* isolate obtained from a Gulf of Mexico beach (**14**). In this study, *Marinobacter* comprised a large proportion of the active community under the oil-amended conditions incubated at 25°C. *Marinobacter* spp. were present at an average relative abundances of 27% under the oil-only condition and 17% under 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 might be due to a variety of factors. One possible explanation for this change in relative abundance is that there were fewer *Marinobacter* cells under the oil-plus-Corexit 9500 condition. Alternatively, since these data were obtained from cDNA libraries, it might be that *Marinobacter* was present yet less active under the oil-plus-Corexit 9500 conditions. A third possible explanation is that *Marinobacter* spp. remained at the same levels (either cells or activity) in both treatments, but other organisms increased in abundance or activity under 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 abundances of all the OTUs classified as *Marinobacter*. Therefore, an examination of the difference of the relative abundances of specific OTUs might provide a more accurate estimation of the effects of Corexit 9500 between treatments.

Eighteen *Marinobacter* OTUs were identified as differentially abundant between treatments. Eight of these *Marinobacter* OTUs showed enrichment under the oil-plus-Corexit 9500 condition and the other 10 were enriched under the oil-only condition relative to the oil-plus-Corexit 9500 condition. This would indicate that, while there were certain *Marinobacter* OTUs that may be sensitive to the addition of Corexit 9500, many of the *Marinobacter* OTUs were stimulated by the addition of Corexit 9500. For example, one OTU was present, on average, at 0.3% and 0.4% in the Corexit 9500-only and the oil-only samples, respectively. However, in the presence of both oil and Corexit 9500, this OTU increased to 2.2%, suggesting that this specific *Marinobacter* population was stimulated by the addition of Corexit 9500 when oil was present. This potential stimulation would be in contrast to a previous report, which found an inhibition of an *M. hydrocarbonclasticus* isolate with the addition of Corexit 9500 (**14**). This discrepancy might be due to the high levels of Corexit 9500 used in the *M. hydrocarbonclasticus* study. In this study, the concentration of Corexit 9500 was 1.12 µg/liter and may not be inhibitory to all *Marinobacter* species.

There were some examples of *Marinobacter* OTUs that exhibited the opposite trend. For example, one OTU comprised 0.7% of the cDNA libraries with Corexit 9500 alone. In the samples with oil, this OTU increased to 21% of the recovered sequences. When oil and Corexit 9500 were present, the abundance of this OTU was only 10% of the library. Thus, there were fewer sequences recovered in the oil and Corexit 9500 treatment than with the oil alone. This 2-fold decrease in recovered sequences might be indicative of an inhibitory effect of Corexit 9500 addition on this OTU. Similar phenomena were observed with many of the other differentially abundant OTUs, with some members of the same genus showing higher abundance under the oil-only versus under the oil and Corexit 9500 conditions and vice versa. However, there were some differentially abundant taxa that were enriched only under one condition. For example, three OTUs classified as *Alcanivorax* spp. were enriched under the oil-only condition compared with that under the oil-plus-Corexit 9500 condition. One of the differentially abundant *Alcanivorax* OTUs was the dominant *Alcanivorax* OTU found in these microcosms. At 25°C, this OTU comprised, on average, 6.7% of the oil-only cDNA communities and 3.5% of the oil-plus-Corexit 9500 communities. This statistically significant difference suggests that in the Corexit 9500-amended microcosms, this *Alcanivorax* sp. was not as competitive. Interestingly, in the 5°C incubation, this same OTU comprised, on average, 56.8% and 59.5% of the active communities in the oil-only and the oil-plus-Corexit 9500 microcosms, respectively. This suggests that at 25°C, there is a possibility that this OTU is inhibited by Corexit 9500; by contrast, at 5°C, the addition of Corexit 9500 had no effect. Therefore, the effect of Corexit 9500 inhibition may be species specific and dependent upon temperature. This is in agreement with recent studies demonstrating that Corexit 9500 did not affect the growth of *A. borkumensis* (J. Liu and T. Hazen, unpublished results).

Conclusions. The goals of this study were to provide additional insights into the effects of Corexit 9500 on the biodegradation of ANS crude oil as well its effects on the community structure of oil-degrading microbes. These results combine to show that biodegradation is enhanced by the addition of Corexit 9500. Our results indicate that microbial community

structure is only impacted by Corexit 9500 addition at 25°C and not at 5°C. Corexit 9500 addition at 25°C had a stronger effect on the active bacterial populations. We have demonstrated that while there were some OTUs negatively impacted by the 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. The results from this study confirm those from previous studies that have shown a positive impact of dispersants on the 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.

MATERIALS AND METHODS

Microcosm setup. Two different enrichment cultures were generated from samples collected near the site of the *Deepwater Horizon* oil spill (**40**). 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 (**19**). 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) (**21**) amended with South Louisiana crude oil, were harvested via centrifugation, and were washed twice with saline to remove oil. The washed cells were resuspended in saline solution containing glycerol (10% final concentration) and stored at −20°C until they were used in the microcosm studies. The cryo enrichment was derived from a sample collected from deep water from the Gulf of Mexico at a depth of 1,240 m and is designed to be representative of a deep-water oil-degrading consortium. The meso enrichment was derived from a surface-water sample from the Gulf of Mexico at a depth of 5 m and is designed to be representative of a surface-water oil-degrading consortium.

In this study, several frozen stocks of the appropriate enrichment were thawed, were combined, and were washed once with twice the volume of saline (to remove glycerol). Aliquots of the final resuspended stock (0.5 ml) were then used to inoculate each of the flasks containing GP2 medium amended with ANS oil, dispersant (Corexit 9500), or ANS oil and dispersant at a volumetric dispersant-to-oil ratio of 1:25 (final Corexit concentration of 1.12 µg/liter). Treatments were incubated aerobically by shaking at 200 rpm at the appropriate temperature (25°C for the meso and 5°C for the cryo enrichments). Sodium azide-killed controls were also prepared to account for abiotic hydrocarbon losses, similar to the setup described by Campo-Moreno et al. (**19**). Experiments were conducted in triplicates per treatment with a total of 100 ml of medium in 250-ml flasks. The contents of flasks incubated for 0, 2, 4, 8, 12, 16, 24, 32, 40, and 48 days were used to measure hydrocarbon residual concentrations and to conduct molecular analyses. Similar measurements were conducted for the cryo enrichment at 56 days.

Hydrocarbon measurements. Subsamples were collected from each flask to determine the concentration of DOSS by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and of hydrocarbons by gas chromatography-tandem mass spectrometry (GC-MS/MS). The procedures used for DOSS and oil extractions and the internal standards

used to normalize for DOSS, total alkanes, and total aromatics are described elsewhere ([19](#), [40](#)). Hydrocarbons are reported as a ratio to the conservative biomarker hopane, which has been routinely used for determining the biodegradation of oil ([41](#)).

DNA/RNA extraction and sequencing. For each time point, samples (1 ml) were transferred from the microcosms to sterile microcentrifuge tubes. Samples were then centrifuged at 10,000 × g for 10 min. Supernatants were decanted without disturbing the cell pellets and the tubes were stored at −80°C until nucleic acids were extracted. Both RNA and DNA were extracted from the frozen cell pellets with an AllPrep DNA/RNA mini kit (Qiagen GmbH) ([42](#)). RNA extracts were further purified with Turbo DNA-free (Ambion) kits according to the routine DNase treatment step. Purified RNA was eluted to a final volume of 30 µl and stored at −80°C. cDNA was synthesized from the purified total RNA extracts using SuperScript III (Life Technologies, San Francisco, CA). RNA and DNA concentrations were measured by Qubit RNA and dsDNA high sensitivity assay kits (Life Technologies) and the Qubit 2.0 fluorometer (Life Technologies). Aliquots (2 µl) of nucleic acid extracts were used in all quantification reaction mixtures. The concentrations of the DNA extracts ranged from 0.05 to 1 ng/µl, while RNA extract concentrations ranged from 2 to 8 ng/µl, with most within 2 to 5 ng/µl. cDNA synthesis was performed using SuperScript III first-strand synthesis kits (Invitrogen) using random hexamer primers according to the manufacturer's instructions. cDNA synthesis was performed the same day as the RNA extraction to maximize RNA yield and to minimize RNA degradation. Aliquots of the RNA extracts were used as the PCR templates to verify the absence of DNA contaminants. An 8-µl volume of RNA was used for the synthesis, generating a final product volume of 21 µl. cDNA was stored at −20°C and excess RNA was stored at −80°C.

The bacterial compositions of each sample were determined via sequencing analysis of the 16S rRNA gene V4 region using the primers 515f/806r as described elsewhere ([43](#)). The PCR assays used to generate the sequencing libraries were performed in 25-µl volumes using the Ex Taq kit (TaKaRa) with 200 nM concentrations (each) of the forward and reverse primers and 2 µl of template nucleic acid. Cycling conditions involved an initial 5 min denaturing step at 95°C, followed by 35 cycles at 95°C for 45 s, 50°C for 60 s, and 72°C for 90 s, and a final elongation step at 72°C for 10 min. Each barcode corresponded to an eight-base sequence unique to each sample. Amplicons were visualized on an agarose gel to confirm product sizes. Aliquots of each amplicon were pooled based on band intensities and sequenced on an Illumina MiSeq benchtop sequencer using 250-bp paired-end kits at the Cincinnati Children's Hospital DNA core facility.

16S rRNA library construction and analysis. The resulting DNA sequences were analyzed using the QIIME version 1.8.0-dev pipeline ([44](#)). Paired-end raw reads were assembled with fastq-join ([45](#)). The assembled sequences were demultiplexed and quality filtered in QIIME to remove reads with Phred scores below 20 (Q19). Chimera detection was then performed on assembled reads with UCHIME ([46](#), [47](#)). Sequences were then clustered into operational taxonomic units ([OTUs] 97% similarity) with UCLUST ([46](#)) using the open reference clustering protocol. The resulting representative sequences were aligned using PyNAST ([48](#)) and given a taxonomic assignment using RDP ([49](#)) 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 (1,670 sequences). Bray-Curtis dissimilarity ([50](#)) weighted and unweighted UniFrac distances ([51](#)) were calculated from the rarefied OTU table with the beta_diversity.py script in QIIME. Several OTUs were originally misclassified as *Methylococcales* species. 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. To test the hypothesis that microbial communities in different treatments were significantly similar to each other and statistically different from other treatments, nonmetric 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, a PERMANOVA ([52](#)) was performed on both the Bray-Curtis and weighted UniFrac matrices with the Adonis function in the vegan package ([53](#)) in R ([54](#)). Samples were grouped according to the treatment. A PERMANOVA was performed using 999 permutations. A PERMANOVA of all three treatments will indicate if there is a significant difference between all three groups. To distinguish which treatments were different from each other, a PERMANOVA was done on subsets of the weighted UniFrac distance matrix that only included samples from two of the three treatments.

The metagenomeSeq package ([55](#)), as implemented in QIIME, was used to identify which OTUs were differentially abundant between treatments for each temperature regime. The metagenomeSeq package provides a more robust normalization method than total-sum scaling or relative abundance. It uses cumulative sum-scaling, in which counts are divided by the cumulative sum of counts up to a percentile determined using a data-driven approach. Furthermore, metagenomeSeq employs the zero-inflated Gaussian to account for undersampling of the microbial community in low-coverage samples. This enables a more robust analysis of differential abundances between samples or conditions. Each treatment was compared with the other treatments incubated at the same temperature, and OTUs that were significantly different (corrected P value < 0.05) in one treatment compared with those of the other two were considered to be differentially abundant.

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