

**Comparative Study to Determine the Biodegradability of Dispersants
at Environmentally Relevant Concentrations**

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ABSTRACT

Chemical dispersant agents reduce the interfacial tension between oil and water, and increase the surface area to volume ratio of oil droplets thus facilitating the biodegradation of spilled oil. Dispersants are composed of surface active molecules known as surfactants and various commercial products contain Dioctyl Sulfosuccinate (DOSS) as the active surfactant ingredient. Since previous laboratory studies were conducted at oil and dispersant concentrations significantly higher (~0.7 g/L oil with Dispersant to Oil Ratio (DOR) of 1:25) than those typically found in field conditions, experiments were conducted at low levels of oil and dispersant (28 µg/L oil with DOR of 1:25) in order to determine the degradation trends at environmentally relevant concentrations. Experiments were conducted using two crude oils (Alaskan North Slope (ANS) and Endicott) and two dispersant products (Corexit 9500 and Finasol OSR 52) to study the biodegradation of dispersants and dispersed oil and oil alone samples were used as controls. Two oil degrading cultures, isolated from the surface (meso) and deep sea (cryo) of the Gulf of Mexico, were enriched

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on crude oil at 25 and 5 °C and were used as the inocula. The biodegradation experiments were performed at 5 °C for 56 days and at 25 °C for 48 days using sterile GP2 artificial seawater as the media. The time series concentration of DOSS, the primary surfactant in the dispersants was monitored using LC-MS/MS in addition to the oil concentration which was measured using GC-MS/MS. Although the initial concentration of DOSS in Finasol OSR 52 was 20% higher than in Corexit 9500, over 95% of the anionic surfactant fraction was metabolized for both types of dispersant products by the end of the experiment at 25 °C while it persisted at 5 °C. The effect of dispersant and oil type on microbial community structure was also analyzed using PCR analysis. Results indicated that the abundance of *Thalassospira* correlated well with hydrocarbon degradation trends. The results from this study significantly expands on our understanding of biodegradation of DOSS, dispersed, and non-dispersed oil and also provides information regarding bacterial community composition.

Keywords: biodegradation, oil spills, dispersant, crude oil, RNA

INTRODUCTION

First generation chemical dispersants (industrial detergents) were primarily used in marine oil spills after the first major tanker catastrophe, the *Torrey Canyon* oil spill, in which large amounts of alkylphenol surfactants were delivered to the contaminated area (Clayton, 1993; Committee on Effectiveness of Oil Spill Dispersants, 1989). The addition of dispersant lowered the interfacial tension between spilled oil and water, accelerate the breakdown of oil slicks, and prevent the resurfacing of small droplets (European Maritime Safety Agency, 2010). Moreover, discussions regarding dispersant utilization in deep-sea condition has come to the forefront after the Gulf of Mexico (GOM) oil spill in 2010.

Corexit 9500, a well-known formula capable of dispersing heavy and weathered oils, is listed on the U.S. EPA National Contingency Plan Product Schedule and stockpiled around the world (Lessard and Demarco, 2010). Finasol OSR 52, another product that is widely stockpiled in Europe and European Free Trade Association countries, has not been completely evaluated for its toxicity and biodegradability. However, the pressing demand for deep-sea drilling requires continuing assessment of biodegradability of dispersants and dispersed oil (Zhuang et al. 2016).

In the aquatic environment, biodegradation is one of the predominant oil removal mechanisms in which bacteria utilize the spilled oil as a carbon source for cell growth and metabolism and thus degrade the oil components. While chemical dispersants increase the bioavailability of the oil to the microbes, the degradation of the dispersants themselves can be a challenge for the microbial community. Several studies assessed the biodegradation of C9500, while only Bergueiro-Lopez et al. (1997) examined the metabolism of Finasol OSR 52 by employing a mixture of bacteria called BIOLEN IG 30. To date, the biodegradability of various

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crude oils dispersed with C9500 have been assessed in laboratory experiments, such as Prudo Bay crude oil (Venosa and Holder, 2007), Macondo crude oil (Wang et al. 2016), and South Louisiana crude oil (Campo et al. 2013). Results varied due to the differences in oil properties and microbial community structure. Only a limited number of studies have been conducted where the biodegradability of dispersed Endicott crude oil was evaluated, and with only one publication evaluating the biodegradation by monitoring total petroleum hydrocarbon concentration under anaerobic conditions (Personna et al., 2014).

The goal of this work was to evaluate the effect of temperature on the biodegradability of dispersants and dispersed oils by using two crude oils (ANS and Endicott) and two dispersants (C9500 and Finasol). Both of the medium crudes utilized in this studies were mined in Alaska and serve as EPA standard reference oils. As the major active surfactant ingredient of C9500 and Finasol dispersant, DOSS was measured as the indicator of dispersant biodegradation in this study. Oil and dispersant in this experiment were under parts per billion level which were close to the concentration of field testing from GOM (Kujawinski et al., 2011). Furthermore, the microbial community structure analysis performed would provide a better understanding on the composition and activity levels of oil-degrading organisms.

METHODS

In order to characterize the biodegradation of DOSS and hydrocarbons, experiments were set up to study the degradation of two crude oils (ANS and Endicott), two dispersants (C9500 and Finasol) and three dispersed oils (ANS dispersed by C9500, ANS dispersed by Finasol and Endicott dispersed by C9500) under two temperature conditions which represented the surface (25 °C) and deepwater (5 °C) environments, respectively.

Cultures and Medium

Two mixed cultures of oil degrading bacteria were provided by EPA's Gulf Ecology Division at Gulf Breeze, FL. The mixed cultures were collected at a depth of 1240 m (crypto) and 5 m (meso) near the Macondo well at the GOM, enriched using crude oil and preserved in 10% glycerol at -80 °C until use. These cultures were added to sterilized GP2 artificial seawater as the media in this experiment, which was prepared by dissolving several mineral salts in deionized water (Bidwell and Spotte, 1985).

Treatments

Dispersed oil treatment: The dispersed oil treatment was prepared in a 2 L baffled flask by adding the crude oil and the dispersant to 1.5 L of GP2 at a volumetric dispersant-to-oil ratio of 1:24 (10 µL dispersant: 240 µL oil). The baffled flask was shaken at 200rpm for 10-minutes, followed by a 10-minute settling period to allow the undispersed oil to rise to the top. After settling, 1 L of the dispersion was drained without disturbing the undispersed oil slick on the water surface. This procedure was repeated two more times which yielded 3 L of total dispersed oil, and it was diluted into 12 L of GP2 (Panels A, B and C of Figure). Subsequently, a series of 250 mL silanized shaker flasks were filled with 100 mL aliquots of the dispersed oil.

Non-dispersed oil treatment: To evaluate the biodegradation of crude oil alone, approximately 4 µL crude oil was directly pipetted into each shake flask containing 100 mL sterile GP2. The final concentration of oil in the samples was approximately 28 µg/L, which was almost the same as the dispersed oil treatment.

Dispersant alone treatment: The dispersant alone treatments were prepared by spiking 20 μL of dispersant into 14 L of GP2 under continuous mixing, to yield a concentration similar to the dispersed oil treatment. After 30 minutes of continuously mixing, 100 mL of the solution was added into 250 mL silanized shaker flasks.

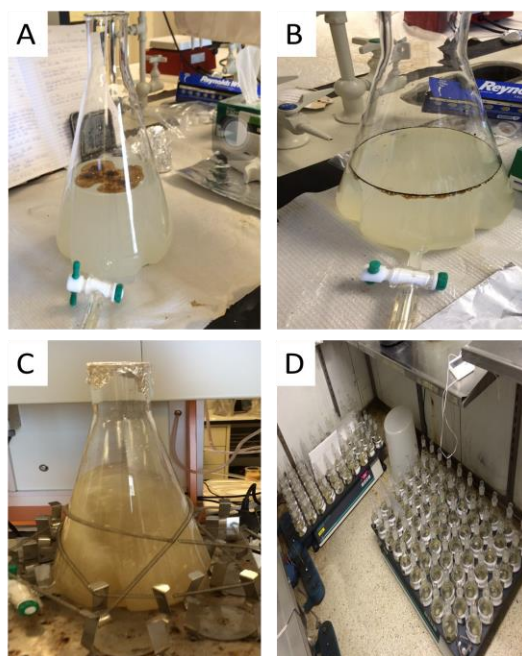


Figure Dispersed Oil Preparation (A, B and C) and Experiment Unit loading (D)

The dispersed oil, non-dispersed oil and dispersant alone treatments were then spiked with 0.5 mL of inoculum per flask with the cryo and meso cultures for the 5 and 25 °C experiments, respectively. After preparation, all the shaker flasks were placed on the orbital shakers in corresponding constant temperature rooms (5 or 25 °C) for the 56 or 48 days, respectively (Panel D of Figure).

Oil component and DOSS analysis

DOSS and oil samples were prepared as described in Campo (2013). According to the ASTM D7730 Standard Method (ASTM D7730, 2011), DOSS was measured by using a 1200 series liquid chromatograph coupled with a 6410 tandem mass spectrometer (LC-MS/MS) from Agilent (Palo Alto, CA). Analysis of oil components was performed on an Agilent (Palo Alto, CA) 7890A GC coupled with an Agilent 7000 mass selective detector triple quadrupole and an Agilent 7693 series auto sampler. It was equipped with a DB-5 capillary column by J&W Scientific (30 m X 0.25 mm, and 0.25 μm film thickness) to achieved chromatographic separation of the alkanes and aromatics. A modified method based on EPA Method 8270D (2007) was followed.

For microbial analysis, 2-mL of sample were collected from the live replicates. The collected samples were centrifuged at 10,000 rpm for 10 min, and the precipitated bacteria were stored in the - 80 °C refrigerator for PCR analysis which determined both the DNA and RNA for the mixed cultures.

RESULTS AND DISCUSSION

Biodegradation of DOSS

Table 1 summarizes the initial concentration, biodegradation rates and removal extents of DOSS, the active anionic surfactant in C9500 and Finasol, with and without crude oils at 25 °C. Although the initial concentration of the primary surfactant (DOSS) in Finasol was around 20% higher than in C9500, over 95% of the anionic surfactant fraction was metabolized for both types of dispersant products by the end of the experiment at 25 °C while it persisted at 5 °C. Thus, the low temperature condition greatly inhibited the microbial uptake of DOSS.

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At 25 °C, biodegradation was fast in the replicates with or without ANS. 96% and 98% of DOSS were degraded for dispersant alone and dispersed oil treatment for two dispersants by the end of experiment, respectively. The presence of ANS remarkably favored the biodegradation first order rate of C9500 by approximately 2.4-fold, but no such effect was observed for the experiment with Finasol and ANS. Besides, DOSS uptake occurred after an acclimation period of 2 days, and removal extents surpassed 98% by day 48 in the presence of Endicott.

Table 1 Summary of Biodegradability of two Dispersants at 25 °C

Treatments	Initial DOSS Concentration ($\mu\text{g/L}$)	Degradation rate (d^{-1})	Removal extent (%)
C9500 alone	203.97 ± 6.98	$- 0.07 \pm 0.009$	96
C9500 + ANS	228.16 ± 11.94	$- 0.16 \pm 0.015$	98
Finasol Alone	280.87 ± 21.19	$- 0.060 \pm 0.008$	96
Finasol + ANS	234.11 ± 5.38	$- 0.068 \pm 0.006$	98
C9500 + Endicott	206.96 ± 4.28	$- 0.077 \pm 0.007$	98

Biodegradation of total alkanes

Table 2 presents the hopane-normalized concentration of total alkanes in dispersed oils. All the targets compounds were normalized by hopane concentration since it was unchanged through the whole experiments in all the treatments. The initial hopane-normalized concentration for 5 °C was almost twice as that of 25 °C because of the oil present in the enrichments. This was confirmed by analyzing the cryo and meso biomass alone, without the addition of any oil. The biodegradation of total alkanes occurred without any lag phase at 25 °C, regardless of the type of dispersed oil, while a 4-day acclimation period was observed at 5 °C. Over 90% of the total alkanes was metabolized by the meso culture by the end of experiment in all the cases.

Table 2 Biodegradability of Hopane-Normalized Total Alkanes in Dispersed Oils at 5 and 25 °C

Temperature	Treatments	Initial Concentration (mg total Alkanes / mg Hopane)	Lag Phase Period (d)	Removal extent (%)
25 °C	ANS + C9500	106.61 ± 3.64	0	92
	ANS + Finasol	113.17 ± 3.69	0	90
	Endicott + C9500	142.91 ± 3.33	0	94
5 °C	ANS + C9500	209.79 ± 7.72	4	85
	ANS + Finasol	220.56 ± 4.30	4	93
	Endicott + C9500	178.42 ± 1.77	4	94

At 5 °C, the presence of Finasol considerably favored the removal of alkanes than C9500 (93% vs. 85%) with ANS. Although, ANS and Endicott crude oil are both medium oil (Table 3), the percentage of total alkanes in Endicott is much higher than ANS crude oil, especially for light alkanes. The remove extent of dispersed Endicott is higher than that of dispersed ANS due to the easier metabolism of short chain alkanes.

Table 3 Physical Properties of ANS and Endicott *

Property	ANS	Endicott
Density, ρ (g/mL)	0.8733	0.8838
Dynamic Viscosity, μ (cP, at 15 °C)	35	120
Kinematic Viscosity, ν (cSt, at 15 °C)	40	134
API gravity	28.24	23.00
Total Alkanes Concentration (mg total Alkanes / mg Crude Oil)	35.52	110.38
of Total PAHs Concentration (mg total PAHs / mg Crude Oil)	20.63	37.46

* General crude oil categories: Heavy (API < 22.3°), Medium (22.3° ≤ API < 31.1°), and Light (API ≥ 31.1°).

* Source: SL Ross Environmental Research, 2010.

Biodegradation of total PAHs

Table 4 presents total PAH degradation for all the dispersed oil treatments at two temperatures. Similar to alkanes, the hopane-normalized total PAH concentration at 5 °C was higher than at 25 °C because of the higher hopane content from the meso cultures. At 25 °C, a notable decline in total PAHs was observed after an acclimation period of 2 days, and, subsequently degraded up to 82% by day 48 in ANS + C9500 treatment. For the other two dispersed oils, PAHs persisted until the end of experiment. Such contrary and unexpected findings is explained in more detail under the microbial community structure section.

At 5 °C, a 12-day lag phase occurred before observable degradation ensued in dispersed ANS treatments, while the cryo culture began degrading PAHs after 32 days for dispersed Endicott. Similar to the alkanes, higher temperature considerably favored the biodegradation of ANS dispersed by C9500 by shortening the lag phase period. At the last sampling event, 21%, 28% and 15% of the initial loaded concentration persisted for ANS + C9500, ANS + Finasol and Endicott + C9500 treatment, respectively. Although Endicott contains more PAHs than ANS (as shown in Table 3), dispersed Endicott was easier to remove owing to the higher percentage of 2-ring compounds which biodegraded faster than 3- and 4- ring PAHs.

Table 4 Biodegradability of Hopane-Normalized Total PAHs in Dispersed Oils at 5 and 25 °C

Temperature	Treatments	Initial Concentration (mg total PAHs / mg Hopane)	Lag Phase Period (d)	Removal extent (%)
25 °C	ANS + C9500	44.38 ± 1.54	2	82
	ANS + Finasol	61.08 ± 2.33	48	3
	Endicott + C9500	53.81 ± 0.14	48	19
5 °C	ANS + C9500	51.17 ± 0	12	79
	ANS + Finasol	63.57 ± 2.25	12	71
	Endicott + C9500	58.55 ± 0.38	32	85

Microbial community structure

As mentioned previously, meso cultures showed significant differences in PAHs uptake between ANS + C9500 and ANS + Finasol experiments which were performed under the same conditions, except for the dispersant product. PCR analysis of these bacterial consortia provided a clearer understanding of the microbial makeup and an in-depth explanation for the inconsistent result.

Table 5 displays the significant species in the active community including *Alcanivorax*, *Pseudoidiomarina* and *Thallassospira* which were abundant in the C9500 experiment but not in the Finasol experiment. In the active community, *Alcanivorax*, one of the predominant hydrocarbon-degrading bacteria in the contaminated seawater, (Cappello et al, 2007; Harayama et al, 2004; Wang et al, 2014) increased from 0.74% to a maximum of 69.3% of total abundance in the C9500 experiment. *Alcanivorax* is a ubiquitous bacteria that was found in the GOM during the DWH oil spill (Kostka et al., 2016). However, the same species in the Finasol experiment was consistently lower at around 20%. Moreover, similar trends were observed by comparing

Pseudoidiomarina and *Thallassospira* (Moghadam et al., 2014). Hence, it is reasonable to infer this loss of active community resulted in the unusual persistence of aromatics at 25 °C.

Table 5 Significant active genes of meso cultures in ANS alone treatment of ANS + C9500 (A) and ANS + Finasol (B) experiments

Species	Experiment	Initial Percentage (%)	Maximum Percentage (%)
<i>Alcanivorax</i>	A	33.13 ± 15.19	69.34 ± 2.10
	B	10.74 ± 3.39	20.58 ± 6.51
<i>Pseudoidiomarina</i>	A	0.15 ± 0.15	21.88 ± 3.88
	B	12.75 ± 1.49	14.93 ± 1.22
<i>Thallassospira</i>	A	0.02 ± 0.03	26.28 ± 2.99
	B	3.92 ± 0.83	0.82 ± 0.69

CONCLUSION

DOSS disappeared promptly at 25 °C, while it persisted throughout the experiments at 5 °C. Additionally, the length of the acclimation period and removal extent were highly impacted by the addition of the dispersant. Similar to DOSS degradation, temperature affected the biodegradation of total alkanes significantly in both the acclimation period and removal extent. Around 80% of the PAHs degraded at 5 °C whereas most PAHs persisted at 25 °C in ANS+Finasol and Endicott+C9500 treatment, which possibly resulted from the loss of several oil degrading microbial species such as *Alcanivorax*, *Pseudoidiomarina* and *Thallassospira*. Although the composition of Endicott considerably differs from ANS, especially the short chain paraffin and 2-ring PAHs, the degradation trends were similar to dispersed ANS, which indicated that the effect of C9500 on the two oils were similar. Since the PAHs persisted at 25 °C, and low activity of

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critical degrading species in meso cultures was observed, it is recommended that different cultures should be evaluated under the same test conditions in future experiments.

Since oil spill is one of the critical marine pollution accidents, the top priorities are to prevent, prepare for, and respond to oil spills, as well as the subsequent recovery of the marine system. This study provided further information of the biodegradation of chemical dispersant and hydrocarbons after the responses which could be good indicator for strategy selection and evaluation.

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