Impacts of a surface washing agent and chemical herder on the aerobic biodegradation of crude oil

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Introduction

Oil spill treating agents **(STAs)**, such as dispersants, surface washing agents, and chemical herders, expand the range of response measures available to decision-makers during oil spills. Due to the reliance on chemical dispersants during the Deepwater Horizon oil spill response, the effects of dispersants on naturally-occuring oil biodegradation in marine environments have been heavily investigated and scrutinized. However, little is known about the fate of other chemical treating agents and their effect on marine microbial communities, including those responsible for oil biodegradation.

The objectives of this 48-day laboratory incubation study were 3-fold:

1) **Measure the impact of two STAs—a surface washing agent and chemical herder—on the aerobic oil biodegradation.**
2) **Determine whether STAs may also be susceptible to biodegradation.**
3) **Describe changes in the resident and active oil-degrading microbial community exposed to weathered oil and STAs.**

Experimental Design & Methods

Weathered Crude Oil: Alaska North Slope **(ANS)** crude oil was weathered by nitrogen gas stripping to achieve a mass loss of 8.7%.

Spill Treating Agents (STAs): Surface washing agent, Cytosol, and chemical herder, ThickSlick 6535, were selected for this study.

Microbial Culture: An oil-degrading culture isolated from a marine beach sample in Maine, USA, was cultured and enriched on **weathered ANS crude** oil in artificial seawater (ASW) amended with sources of nitrogen and phosphorus (Spotte, et al. 1984). Frozen aliquots of this culture served as the initial inoculum in this study and were incubated on a shaker for 4 days before subsequent addition to the serum bottles.

Experimental Design: Microcosms were constructed in glass serum bottles containing artificial seawater with additions as described in **Table 1**. The inoculum was added to achieve a 5% (v/v) cell concentration of the enriched culture. In the applicable treatments, spill treating agent was added to the microcosms at a ratio of 1:2, treating agent to oil. To account for potential abiotic loss or transformation of oil and STAs, additional controls (not shown) included killed-controls (sterilized with sodium azide), inoculum-unamended treatments, and a treatment containing only inoculum in ASW. Treatments requiring sacrifical sampling were constructed in triplicate per sampling event. The serum bottles, with a total internal volume of 242 ml, were sealed with PTFE-lined stoppers and aluminum caps. The bottles were placed horizontally on an orbital shaker set to 150 rpm, at 20 °C in the dark, and sampled over a 48-day period.

Table 1. Experimental layout.

Treatment	ANS (ul)	CytoSol (ul)	ThickSlick (ul)	Inoculum (ml)	ASW+nutrients (ml)
ANS	80	-	-	4	156
ANS + CytoSol	80	40	-	4	156
ANS + ThickSlick	80	-	40	4	156
Cytosol	-	40	-	4	156
ThickSlick	-	-	40	4	156

Respiration: Carbon dioxide (CO_2) and oxygen were monitored regularly in the headspace of microcosm bottles using gas chromatography (GC) with a thermal conductivity detector (TCD) in-series with a methanizer/flame ionization detector (FID). Oxygen was quantified to ensure aerobic conditions within the microcosm were maintained throughout the incubation period. If oxygen fell below 10% by volume, the headspace was aseptically flushed with air to return oxygen concentrations to atmospheric levels.

Total Organic Carbon (TOC) content of ANS and STAs: US EPA SW-846 Method 9060A.

Biomass: Growth of heterotrophic bacteria was quantified using Idexx EasyDisc PCA Tests.

Oil Hydrocarbons: Following liquid-liquid extraction, gas chromatography-mass spectrometry (GC-MS) was used to detect and quantify a suite of petroleum hydrocarbons, including alkanes and polycyclic aromatic hydrocarbons, using U.S. EPA Method 8270D.

Microbial Community: Nucleic acids (DNA and RNA) were extracted from aqueous samples using the QIAGEN AllPrep Bacterial DNA/RNA/Protein Kit. Complimentary DNA (cDNA) was synthesized from the purified RNA extracts using Invitrogen SuperScript IV First-Strand synthesis kit using random hexamer primers. The DNA extracts and newly synthesized cDNA were sequenced using Illumina MiSeq targeting the V4 region of the 16S rRNA gene (primers 515F & 806R) by Cincinnati Children's Hospital Medical Center Genomics Sequencing Facility. Sequences were analyzed using the Mothur bioinformatics pipeline (Schloss, et al. 2009). Taxonomic barcharts and nonmetric multidimensional scaling (NMDS) plots were generated in R (V. 4.3.0) using the Phyloseq package (V 1.46.0) and Permutational multivariate analysis of variance (PERMANOVA) statistics were performed using the R Vegan package using Adonis (V 2.6-4).

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Results and Discussion

Respiration and Microbial Growth

- The oil and STAs within the microcosms serve as the sole sources of carbon. Carbon dioxide production is a measure of biological respiration and mineralization (complete biodegradation) of these compounds.
- Over a 48-day period, total CO_2 production ranged from 260 µmol (ANS) to 740 µmol (ANS+CytoSol) CO_2 (**Fig. 1**).
- In the treatments containing only one carbon source, biological respiration is ranked as follows: CytoSol > ThickSlick > ANS.
- Percent Total Organic Carbon of Substrates: ANS (84.8%) > Cytosol (78.1%) > ThickSlick (64%)
  - *The total volume added of STA added (40 µl) was half that of ANS (80 µl) and with lower TOC content, but more CO_2 was produced compared to ANS alone; STAs are readily bioavailable and biodegradable by the oil-degrading microbial community used in this study.*
- Interestingly, higher levels of CO_2 were produced in treatments containing both ANS and STA than the sum of the individual treatments containing only one exogenous carbon source (**Fig. 1**)
  - *The dashed lines in Fig. 1 represent the expected production within the microcosms amended with both oil and STA. The observed production in ANS+Thickslick was about 12% higher than the expected production, and ANS+Cytosol was 17% higher than expected, suggesting a synergistic mechanism at play.*
- Heterotrophic bacteria increased in all treatments containing an exogenous carbon source (**Fig. 2**). Viable cells increased by 1 order of magnitude with the addition of ANS, and by 2 orders of magnitude in treatments containing STA.
  - *Addition of CytoSol and ThickSlick stimulate growth of heterotrophic microorganisms.*

Oil Hydrocarbons

- As shown in **Figs. 3a** and **3c**, the addition of spill treating agent induced a short lag in n-alkane degradation from Day 0 to Day 5. However, the rate of n-alkane degradation was faster in ANS+CytoSol and ANS+ThickSlick from Day 5 to Day 10 compared to the treatment with only ANS.
  - *The initial lag may be caused by a disruption in the functioning microbial community due to the addition of STA. However, treating agents may enhance the bioavailability of the oil compounds or prime specific alkane-degrading members of the microbial community, boosting degradation rates and mineralization efficiency (i.e., more CO_2 produced per mol substrate)—see **Figs. 3a** and **c**, and **Fig. 1***
- At Day 14, 93% and 91% of n-alkanes had biodegraded in ANS and ANS+ThickSlick, respectively (**Fig. 3a**); however, in ANS+CytoSol, higher molecular weight n-alkanes (n-C_{30-35}) persisted with 18% remaining, slowly declining over the remaining 26 days (**Fig. 3c**).
  - *CytoSol has a composition similar to biodiesel, comprised of C_{16-19} fatty acid methyl esters (FAMEs) derived from vegetable oils. These partially oxidized hydrocarbons are easier to degrade than straight-chain alkanes.*
- Degradation of Polycyclic Aromatic Hydrocarbons (PAHs) are not impacted by the addition of CytoSol, as no lag period is observed, and the total mass loss of PAHs is comparable to that of ANS-alone (**Fig. 3b**).
- In the latter part of the study, PAHs and alkanes decline further, coinciding with the gradual increase in CO_2 production measured in all treatments containing ANS from Day 32 onward (**Fig. 1** and **3b**). At the conclusion of the study, an average of 36% total PAHs remained among the treatments.
- EPA's High Priority Pollutant PAHs, designated due to known and potential toxicity, are below detection limit by Day 5 in ANS and ANS+CytoSol, and by Day 7 in ANS+ThickSlick (**Fig. 3d**).
  - *Although ThickSlick impacts PAH degradation, the herding agent was applied at a ratio of 1:2 with ANS; in a real-world scenario, ThickSlick would be used in ratios on the order of 1:500 to 1:1000. In this case, it is unlikely that ThickSlick would have any measurable impact on the naturally-occuring degradation of PAHs in-situ.*

Microbial Community

Table 2. Effects of treatment on selected microbial community members.

Taxonomic Classification	Treatment Outcome	Metabolic Capacity or Relevance
Altererythrobacter	Comprises up to 16% of community in all ANS-containing treatments. Dramatic decline (< 2%) in STA-only.	Common marine bacterium capable of degrading alkanes and PAHs.
Rhodobacteraceae	7-fold increase in resident community in all treatments containing ANS. Only slight enrichment in STA-only.	Prominent marine heterotroph found in oil-polluted waters. Known to degrade straight-chain and aromatic hydrocarbons.
Rhodococcus	Greatly enriched in STA-only treatments	Metabolically versatile; capable of degrading BTEX, high molecular weight PAHs, C_{5-16} alkanes and C_{1-12} alcohols.
Sphingopyxis	Declined markedly in Cytosol	Genus known for degrading a variety of environmental contaminants, including PAHs. Decline likely due to PAHs are not present in CytoSol, and enrichment of other prominent groups.
Gordonia	Heavily enriched in CytoSol	Metabolizes straight and branched alkanes and other long-chain organic compounds similar to the FAMEs present in CytoSol.
Methylophaga	Heavily enriched in CytoSol	May act as a secondary degrader, taking advantage of abundant, simpler degradation metabolites as a carbon source.

- All treatments promoted shifts in the metabolically diverse, oil-degrading, bacterial community (**Table 2** and **Fig. 4**).
- Bray-Curtis NMDS analysis revealed distinct clustering of the resident and active microbial communities (**Fig. 5**). PERMANOVA revealed the resident and active microbial communities (DNA and RNA) were statistically different from each other (Pr>F= <0.001***) when grouped by treatment.
- *Gordonia* prefers FAMEs or out-competed n-C_{30-35} alkanes-degrading bacteria, explaining the recalcitrance in the ANS+CytoSol treatment (**Fig. 3c**).
- ThickSlick (65% surfactant Span 20 and 35% solvent 2-ethyl butanol), is easily consumed by the inoculum. Microbial community analysis does not capture if substrate preference or competition by any group is responsible for the slight delay in PAH degradation (**Figs. 3b** and **3d**).
- Relative magnitude of community members is different between the DNA and RNA (as cDNA) fractions.
  - *Hoeflea* is equally represented in the resident microbial communities (DNA) across treatments including the original inoculum (~23%) but is underrepresented in the active portion (RNA), particularly in the ANS-only treatment (6%).
  - Similarly, by Day 7 in the Cytosol-only treatment, *Methylophaga* and *Gordonia* make up about 23% and 9%, respectively in DNA, but their predominance is swapped in the RNA, with *Methylophaga* constituting only 6% of the community and *Gordonia*, 21%.

Conclusions

- ***Treating agents investigated here may drive microbial community structure, due to biodegradability, but pose little risk to oil-degrading communities in-situ, potentially enriching for specific microbial groups that promote complete biodegradation.***
- ***The molecular diversity and significance of some members within the microbiome may be misinterpreted by exclusively targeting the 16S rRNA gene. Sequencing 16S rRNA gene transcript alongside the 16S rRNA gene provides a more robust understanding of the ecology and dynamics of contaminant-degrading microbial communities.***

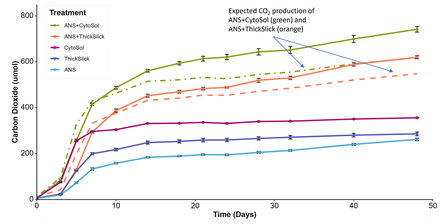

Figure 1: Cumulative carbon dioxide production.
Error bars represent standard error of three replicate microcosms. Expected concentration is the sum of the related individual treatments.

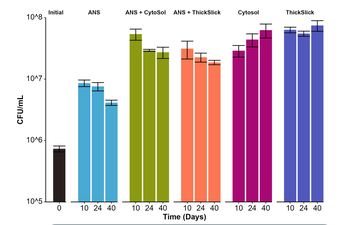

Figure 2: Growth of heterotrophic bacteria.
Error bars represent 1 standard deviation of triplicate plates of pooled triplicate microcosms.

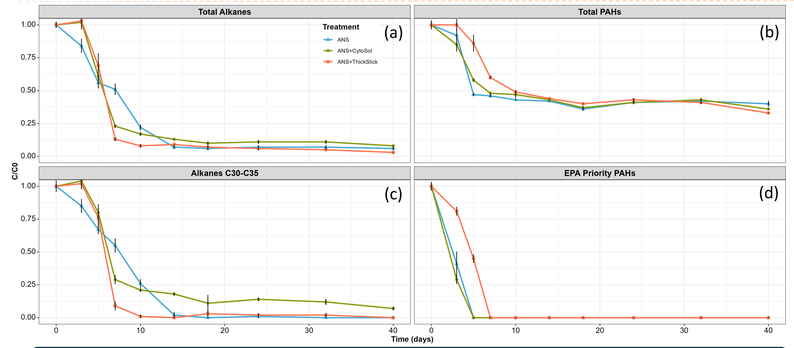

Figure 3: Biodegradation of oil hydrocarbons.
Individual analytes normalized to hopane and normalized to the starting concentration. Error bars represent standard error of three replicate microcosms.

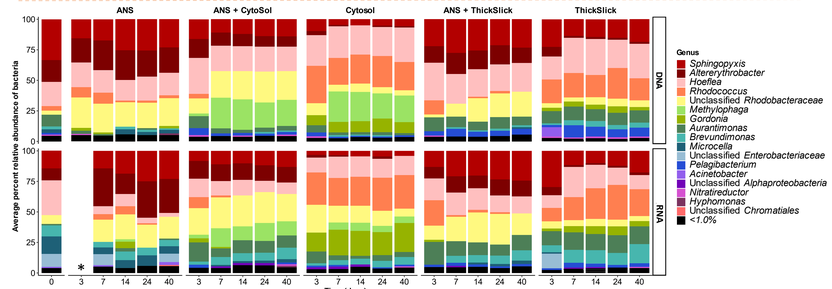

Figure 4: Relative abundance of resident and active bacterial groups, as DNA and RNA, respectively.
Mean of triplicate samples, grouped by genus and treatment type. *Data missing due to insufficient RNA recovery

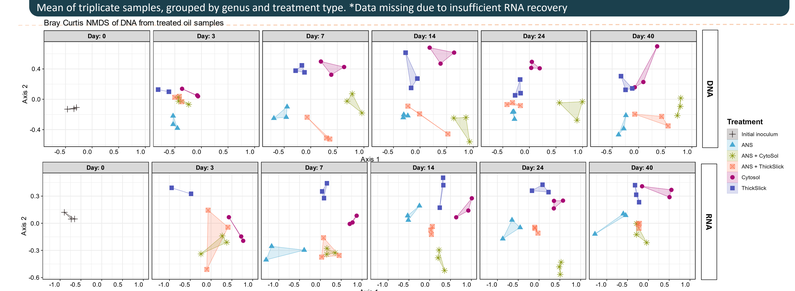

Figure 5: Bray-Curtis NMDS Plots.
Ordination represents clustering of resident (DNA) and active (RNA) bacterial communities.

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