ACS Sustainable Chemistry & Engineering

Research Article

Cite This: ACS Sustainable Chem. Eng. 2019, 7, 14490−14499

pubs.acs.org/journal/ascecg

Biofilm Formation by Hydrocarbon-Degrading Marine Bacteria and Its Effects on Oil Dispersion

Marzhana Omarova,[†] Lauren T. Swientoniewski,[‡] Igor Kevin Mkam Tsengam,[†] Diane A. Blake,[‡] Vijay John,[*,†] Alon McCormick,[§] Geoffrey D. Bothun,[∥] Srinivasa R. Raghavan,[⊥] and Arijit Bose[∥]

[†]Department of Chemical and Biomolecular Engineering, Tulane University, 300 Lindy Boggs Building, New Orleans, Louisiana 70118, United States

[‡]Tulane University School of Medicine, 1430 Tulane Avenue, New Orleans, Louisiana 70112, United States

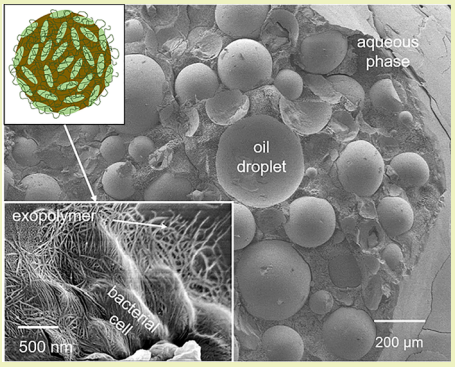
[§]Department of Chemical Engineering and Material Science, University of Minnesota, 421 Washington Avenue SE, Minneapolis, Minnesota 55455, United States

[∥]Department of Chemical Engineering, University of Rhode Island, 51 Lower College Road, Kingston, Rhode Island 02881, United States

[⊥]Department of Chemical and Biomolecular Engineering, University of Maryland, College Park, 4418 Stadium Drive, Maryland 20742, United States

Supporting Information

ABSTRACT: Biodegradation of oil by marine bacteria is a significant pathway to oil spill remediation. Marine hydrocarbon degrading bacteria are known to form biofilms consisting of exopolymer and interconnected bacterial cells. This work indicates that microbial biofilm aids in the stabilization of dispersed oil droplets through the formation of biofilm at the oil−water interface and is therefore an environmentally benign and sustainable method to aid dispersion of spilled oil. Using a model hydrocarbon degrading organism *Alcanivorax borkumensis,* we show, through a combination of optical and high-resolution cryogenic scanning electron microscopy, that these microbes sequester into biofilm at the oil−water interface. We show that the bacterial culture incubated for 3 days and containing biofilm can disperse oil slicks moderately well (40−50%) as estimated by the baffled flask test and can thus be used as an environmentally benign response to oil spills. The dispersion occurs through bacterial adsorption at the oil−water interface together with the aid of naturally secreted biosurfactants that lower the oil−water interfacial tension by a factor of 2 to around 23 mN/m. When the bacterial culture is incubated for a week, the presence of biofilm at the interface can have a hindering effect at oil dispersion through formation of a rigid interfacial layer of biofilm. We show that the dispersion effectiveness of the commercial dispersant Corexit 9500A decreased approximately 25% in the presence of a mature microbial biofilm at the interface. Hexadecane biodegradation by the microbial culture was estimated, and it was found that approximately 90% of hexadecane was degraded in the period of 5 days. This work provides a comprehensive view on marine microbial biofilm from a detailed characterization at the formation stage to the overall role in the context of oil spill dispersion and further biodegradation. Bacterial biofilm and biosurfactants represent fully environmentally sustainable and natural materials for oil spill dispersion.



KEYWORDS: *Alcanivorax borkumensis, exopolymer, Anadarko crude oil, baffled flask test, oil dispersion, oil−water interface, biodegradation*

INTRODUCTION

Bacteria are known to form aggregates of cells in the form of mats, flocs, or biofilms that are essential for their survival.[1−3] Biofilms are composed of microbial cells and extracellular matrix that can make up to 90% of the weight of biofilm.[1] The extracellular matrix is a conglomeration of polymers in which cells are immobilized, and it is typically referred to as extracellular polymeric substances (EPS). EPS typically consist of polysaccharides, proteins, nucleic acids, lipids, and humic substances,[4] all of which determine the hydrophobicity, biodegradability, and adsorption properties of the biofilm.[5,6] The microbial EPS is a multipurpose structure; it serves as anchoring material for the microbes to attach onto surfaces,[7] helps regulate the mass transfer of nutrients and waste products,[6] provides protection from the environment with regard to temperature and osmolarity, adsorbs metals[8] and different organic compounds,[9] and is also the building material for the three-dimensional architecture of the biofilm that

Received: April 6, 2019
Revised: July 16, 2019
Published: July 29, 2019

DOI: 10.1021/acssuschemeng.9b01923
ACS Sustainable Chem. Eng. 2019, 7, 14490−14499

ensures its mechanical stability.[10] The existence of microbial biofilm and EPS has been known since the early 20th century,[11−13] and current scientific interest lies in prevention and destruction of microbial biofilm in public-health related issues.[14] There is also significant interest in utilizing EPS for wastewater treatment as low-cost flocculants[15] and heavy metal sorbents.[16,17]

Microbial biofilms play an important role in the marine environment, where microbes sequestered in the biofilm matrix ensure the proliferation of bacteria in seawater, and facilitates their adhesion to substrates.[18] Biofilms in the marine environment are complex structures, where dynamic cooperation and competition processes between various microbial species affect the structure of the biofilm and the abundance of species in it.[11] Coaggregation of bacterial biofilms and the influence of nonmicrobial microorganisms present in the marine environment continuously shape communities in the biofilm.[11,12] Marine biofilms are of significant relevance. For example, specific bacterial species such as *Pseudomonas, Sphingomonas, Flavobacterium*. and their biofilms are undesirable due to their fouling of surfaces of ships, offshore platforms, and underwater equipment.[14,19] On the other hand, many bacterial species are natural hydrocarbon degraders (*Alcanivorax, Cycloclasticus, Fundibacter, Oleispira*) and they are an essential aspect of cleaning up the ocean water after oil spills.[20] In addition, there is an increased interest in using microbial biosurfactants as an environmentally friendly method for enhanced oil recovery.[21,22]

Oil spills are major disasters affecting human-environmental systems, as the examples of the Exxon Valdez[23] and the Deepwater Horizon[24] have shown.[25] While there are different methods of response and remediation, such as collection by skimmers[26] and in situ burning,[27] the application of chemical dispersants is a convenient large-scale response that has been deployed in many cases.[28] Dispersants are very effective at dispersing floating oil into small droplets that remain stable in the water column. However, their use is still highly controversial due to concerns regarding their potential toxicity to marine organisms.[29] This has necessitated development of alternative dispersing agents that are more environmentally friendly. One direction of this work takes advantage of emulsion stabilizing agents that are native to the marine environment, such as bacterial cells and related biosurfactants.

Bacterial cells can stabilize emulsions by adsorbing to interfaces in a manner similar to colloidal particles. Emulsions in which solid particles stabilize droplets of one immiscible phase in the other are known as Pickering emulsions, and the solid particles acting as stabilizers are Pickering emulsifiers.[30] Both bacterial cells as well as the solids produced by them as a part of biofilms can stabilize emulsions. Gong et al.[31] reported the use of dodecanol-modified bacterial cells as a Pickering emulsifier. In addition, the biosurfactant secreted by bacteria can impact emulsion droplet size and stability. Thus, several reports propose the use of bacterial biosurfactants as a possible alternative to the commercial chemical dispersants.[32−34] The recent work on marine EPS by Schwehr and co-workers found that EPS constituents may be more efficient at stabilizing emulsions at lower concentrations compared to Corexit.[35] Microbial biofilms could effectively be a method of lowering interfacial tension due to biosurfactant and stabilization by steric hindrance due to both the bacterial cells and the exopolymer produced.

Our work is based on the hypothesis that the marine microbial biofilm has a role to play in the dispersion of crude oil in seawater especially after aging and that this effect is separate from the normal weathering effect of oil. The direct concept is that hydrocarbon-degrading bacteria generate biosurfactants that help reduce the oil−water interfacial tension to form droplets upon agitation through natural wave action. At the same time, the generation of EPS forms a barrier at the oil−water interface and helps stabilize droplets against rapid recoalescence. A model hydrocarbon degrading marine bacterium *Alcanivorax borkumensis* was used to test the working hypothesis of droplet stabilization by biofilm. This species of hydrocarbon-degrading marine bacteria produces both biosurfactant and generates biofilm within which it is sequestered at the oil−water interface.[36−38] Recent pioneering work by Godfrin and co-workers[39] clearly showed distinctions in the aggregation morphologies of *A. borkumensis* when grown in clean conditions and when grown in the presence of an oil phase. These authors indicated significant clustering at the oil−water interface and the generation of biosurfactant that reduced the oil−water interfacial tension.

Our work is distinct in that we study the generation of biofilm at the oil−water interface and simply consider the bacterial cells to be embedded in the biofilm. In the literature, microbes are studied in the context of oil biodegradation and as colonizers of preformed droplets.[40,41] However, the formation of stable oil droplets in seawater through microbial biofilms has received insufficient attention. Thus, in this paper, we describe the formation of the biofilm of *A. borkumensis* and its role in the stabilization of the oil−water interface to prevent coalescence of oil droplets. This is an entirely natural system of emulsification and emulsion stabilization and could be a benign and sustainable aid to oil dispersion in marine environments. A key aspect to the work is the use of cryogenic scanning electron microscopy (cryo-SEM) to visualize the formation of biofilm, the details of biofilm morphology at the oil−water interface, and aspects of biofilm anchoring to the oil−water interface.

EXPERIMENTAL SECTION

Materials. *n*-Hexadecane and resazurin sodium salt were obtained from Sigma-Aldrich. *n*-Hexadecane was filtered twice through a 0.22 μm syringe filter before use. Deionized (DI) water generated by an ELGA reverse osmosis water purification system (MEDICA 15BP) with a resistance of 18.2 MΩ·cm was used in all experiments. Anadarko crude oil was obtained from the Bureau of Safety and Environmental Enforcement (BSEE) facilities at Ohmsett, New Jersey. *Alcanivorax borkumensis* (ATTC-700651) was obtained from the American Type Culture Collection (Manassas, VA). Freeze-dried cultures were reconstituted according to ATCC instructions, using Difco Marine Broth and agar obtained from Thermo-Fisher (Fairlawn, NJ). An ONR7a medium was used to simulate the composition of seawater[42] and prepared according to the published protocol.[43] Gibco antibiotic-antimycotic was purchased from Thermo-Fisher. Corexit 9500A was obtained from Nalco Environmental Solutions LLC and is hereafter designated as Corexit.

Bacterial Growth Experiments. A liquid culture of *Alcanivorax borkumensis* was grown on hexadecane (1% v/v) in ONR7a synthetic medium. When the OD600 of the inoculum reached 0.5 indicating the exponential phase, the culture was washed by centrifugation with ONR7a medium to get rid of traces of hexadecane and used for experiments. Inoculum at a ratio of 1:100 was added in all experiments. To monitor the bacterial growth, *A. borkumensis* inoculum was introduced into 3 mL of ONR7a medium in 10 mL polystyrene culture tubes, Anadarko crude oil was added at 1:100 v/v

DOI: 10.1021/acssuschemeng.9b01923
ACS Sustainable Chem. Eng. 2019, 7, 14490−14499

ratio, and the tubes were kept on a shaker at 150 rpm at 30 °C. Samples were taken in triplicate for every time point to be analyzed. Bacterial cell growth was monitored by measuring absorbance at 600 nm (Shimadzu UV-1700) and by resazurin assay.[44] A 0.4 mL aliquot of the collected samples was reacted with 40 μL of resazurin solution in ONR7a medium. Resazurin conversion was left to proceed for 4 h, after which 200 μL of serially diluted samples in 96-well plates was analyzed for fluorescence.

Bacterial Biofilm Characterization. A Hitachi S-4800 field emission scanning electron microscope with the operating voltage of 3 kV was used to obtain cryogenic SEM images of emulsions and bacterial biofilm. The samples were placed into rivets mounted onto the cryo-SEM sample holder. The samples were then plunged into slushed liquid nitrogen for vitrification. This was followed by fracturing at −130 °C using a flat-edge cold knife and sublimation of the solvent at −95 °C for 15 min to etch the sample. The temperature was lowered back to −130 °C, and the sample was then sputtered with a gold−palladium composite at 10 mA for 132 s before imaging. The fractured surface of an emulsion aliquot was imaged with a focus on large emulsion droplets, as the smallest droplets are difficult to distinguish from the continuous phase.

Interfacial Tension Measurements. The interfacial tension between *n*-hexadecane and an aqueous phase based on the ONR7a medium was measured by the pendant drop method using a standard goniometer (Rame-Hart, model 250 with DROPimage software). A 20 μL drop of aqueous sample was injected into 5 mL of hexadecane outside phase. The injected sample was filtered twice through a 0.22 μm syringe filter before injection. The droplet was left to stabilize for at least 10 min or until the variation in two consecutive (interval of 10 s) interfacial tension measurements was less than 0.01 mN/m.

Dispersion Effectiveness Measurements. We used a baffled flask test[45] that was adapted for bacterial growth and biofilm formation. A 1 mL sample of inoculum was introduced to 100 mL of ONR7a medium amended by 100 μL of Anadarko crude oil. The abiotic controls included antibiotic−antimycotic added at a 1:100 volume ratio. The flask was left to swirl gently at 30 rpm. The flask was then used for dispersion effectiveness measurement as described in the published protocol.[45] The percentage light transmittance through the emulsion was then measured on a UV−vis spectrophotometer (Shimadzu UV-1700) at a wavelength range of 340−400 nm, and the UV Probe software (version 2.32) was used for data analysis.

Emulsion Characterization. Aliquots of emulsions were used for emulsion droplet size characterization using an Olympus IX71 microscope in the bright field mode. The average droplet diameter of the emulsions (at least 300 droplets per sample) was estimated by ImageJ software.

Biodegradation Measurements. The experimental setup was analogous to the one used for growth curve monitoring. Anadarko crude oil was doped with anthracene as the internal standard (3 mg/mL) before oil was added to the bacterial culture. Crude oil was additionally doped with hexadecane to 5 mg/mL. After certain time periods, samples were used to assess biodegradation. The oil fraction was extracted by dichloromethane and hexane mixture (1:1 v/v), and the extracts were used to quantify biodegradation of *n*-hexadecane in Anadarko crude oil by GC-FID (Agilent 7820A, HP-5 column). The program used was as follows: start at 50 °C, hold for 2 min, temperature increase was 10 °C/min until the temperature reached 280 °C. The detector temperature was kept at 300 °C.

RESULTS AND DISCUSSION

Cell Growth Characteristics and Biosurfactant Production. *A. borkumensis* is a predominant species in the marine consortium surrounding an oil spill,[46] and its ability to degrade aliphatic hydrocarbons makes it a suitable model for studies on bacterial growth and biodegradation.[38,46,47] Figure 1 shows growth curves of *A. borkumensis* bacterial cells in ONR7a synthetic medium containing nitrogen, phosphorus, and iron amended with Anadarko crude oil at a 1:100 v/v ratio.

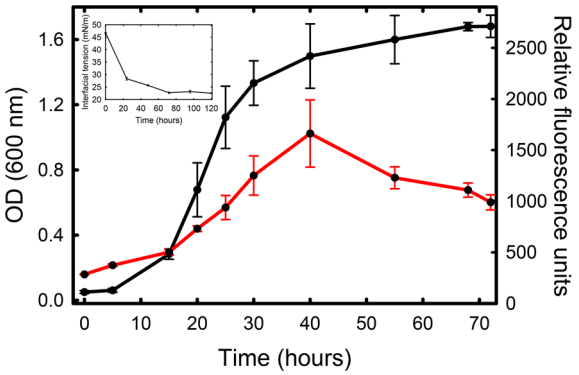


Figure 1. Growth curves of *A. borkumensis* as measured by absorbance at 600 nm (black curve) and resazurin viability assay (red curve). Hexadecane-ONR7a medium interfacial tension changees with the growth of *A. borkumensis* culture (inset) and generation of biosurfactant.

Bacteria were incubated in culture tubes that were gently shaken on an orbital shaker at 150 rpm. At specific time points the samples were collected to measure the optical density (OD) and to carry out the resazurin assay. The latter method generates a response from live cells and is contrasted with the OD curve to show that the bacterial population increases rapidly only in the first 2 days of incubation. It should be noted that the OD measurements are based on turbidity and include contributions from both cells and biofilm filaments which add to the turbidity. In contrast, the resazurin assay which involves cell-mediated reduction of resazurin to resorufin[44] clearly measures the viable cells in the system. Thus, the resazurin assay provides information about the presence of live and active bacterial cells. We see a sharp decline in the response coming from the viable cells after 40 h which is due to the drop in pH in a confined system.[38] The drop in the number of viable cells perhaps occurs due to acidification of the growth medium as observed by Scoma and co-workers[48] and Barbato and co-workers[49] and has been attributed to metabolites released into the medium. The generation of biosurfactant is particularly noteworthy as the oil−water interfacial tension (inset to Figure 1) decreases with time. We note that the interfacial tension measurements were done using the pendant drop method of a droplet of the cell culture in a bulk clear hexadecane phase (rather than the dark Anadarko crude oil) to enable visualization of the water droplet.

The inset to Figure 1 shows that generation of biosurfactant accompanies bacterial growth, and the culture is able to reduce interfacial tension (between the aqueous ONR7a medium and hexadecane) from 47 to 23 mN/m over 3 days of incubation. The lowest hexadecane-ONR7a interfacial tension observed in bacterial samples is 23 mN/m. A decrease in interfacial tension beyond 3 days is insignificant possibly relating to the drop off in the number of live cells that generate biosurfactant. Similar observations of interfacial tension reduction were noted by Godfrin and co-workers.[39]

Bacterial Biofilm Growth at the Oil−Water Interface. Figure 2 shows characteristics of *A. borkumensis* attachment and the biofilm, after the oil−water interface of the Anadarko crude oil has been colonized by the organism in an experimental setup similar to the one reported in Figure 1. We note that the system represents a gently shaken culture on an orbital shaker with no effort made to disrupt the oil layer. Thus, the direct photographic image of the interface (Figure

DOI: 10.1021/acssuschemeng.9b01923
ACS Sustainable Chem. Eng. 2019, 7, 14490−14499

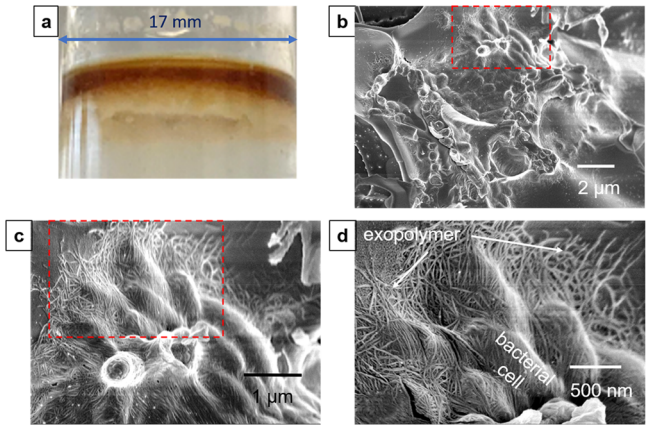


Figure 2. *A. borkumensis* biofilm: (a) digital photograph of *A. borkumensis* biofilm grown at the crude oil-ONR7a interface in a culture tube; (b) cryo-SEM showing bacterial cells and exopolymer fibers around them after 4 days of incubation; (c) higher magnification cryo-SEM image showing the bacterial cells are covered in exopolymer and interconnected by it; (d) cryo-SEM showing individual bacterial cells and exopolymer fibers on and around the cells. Panels b–d are based on the same image but with higher magnification.

2a) reveals a mucus-like layer below the oil representing the microbial biofilm containing bacterial cells and exopolymer, that is typical of microbial activity at surface oil layers.[50] The appearance of the thick biofilm at the interface occurs over 3–4 days after the inoculum is introduced to oil amended ONR7a growth medium. The panel of cryo-SEM images performed by sampling the biofilm followed by vitrification (Figure 2b–d) shows a progression from low to high magnification resolving the microstructure of the biofilm. The remarkable clarity of individual fibers of biofilm can be observed through the high resolution cryo-SEM.

Microbial biofilm growth at the oil–water interface was additionally monitored on an immobilized oil surface with the experimental setup as shown in Figure 3a. For this experiment, a funneled capillary (capillary outer diameter = 0.1 mm) was chosen to simulate a static oil–water interface. A short, closed capillary was first filled with ONR7a medium and subsequently 1 μL of crude oil was injected into the medium using a narrow bore needle inserted through the funnel. The capillary was further placed horizontally onto a Petri dish to allow microscopy imaging. The Petri dish was filled with more ONR7a medium to cover the capillary and allow bacterial access to oil from the funnel side. Bacterial inoculum was then introduced to the Petri dish to simulate gradual colonization of the oil–water interface, and the system was imaged with an optical microscope at specific time intervals. Figure 3 panels b–d show the changes as *A. borkumensis* cells form biofilm at the interface. The small dots in the micrograph represent the bacterial cells but these are hard to visualize in the static micrograph of Figure 3c; the video provided in the Supporting Information (Video S1) shows bacterial movement in the aqueous phase and close to the oil–water interface. After 24 h, bacterial cells closest to the interface undergo a significant loss in motility as they become sequestered in the biofilm. Figure 3d indicates that after 72 h, clearly visible biofilm has formed. In the duration of this experiment, the biofilm grows to form a mat at the interface of approximately 50 μm thickness (Supporting Information, Figure S2). This biofilm mat appears to be a mechanical barrier at the interface. It is known that bacterial biofilms grown on oil–water interfaces, such as those of *Pseudomonas aureginosa*,[51] *Pseudomonas putida*,[52] and *Marinobacter hydrocarbonoclasticus*[53] exhibit a degree of elasticity and stiffness.

The Role of Biofilm in Oil Dispersion. We posit that *A. borkumensis* biofilm as well as bacterial cells in the culture can stabilize emulsion droplets created by the biosurfactant-induced reduction in the interfacial tension and can thus be used to disperse oil. Figure 4 describes the experimental design for dispersion testing. *A. borkumensis* inoculum was introduced into the growth medium, after which oil (100 μL) was layered at the surface of the medium (100 mL) resembling a slick. The resulting thin oil layer was then allowed to be colonized by bacteria. During bacterial growth and interface colonization the flasks were kept gently swirling on an orbital shaker (30 rpm). At the end of a specific time period, the flasks were shaken vigorously for the baffled flask test (200 rpm for 10 min following the EPA protocol[45]). Our experiments therefore consider dispersion effectiveness in the absence of any added chemical dispersant where dispersion is the consequence of bacterial biosurfactant and/or biofilm formation.

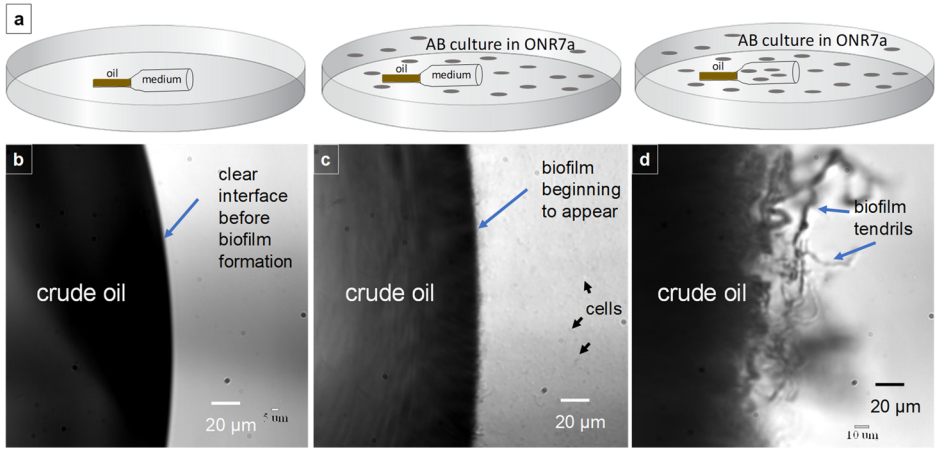


Figure 3. (a) Schematic of the experimental setup for monitoring biofilm growth. Crude oil-ONR7a medium interface in the capillary: (b) the interface before biofilm growth, (c) the interface is less sharp after 24 h due to bacteria populating the oil surface, (d) significant biofilm growth at interface after 72 h.

DOI: 10.1021/acssuschemeng.9b01923
ACS Sustainable Chem. Eng. 2019, 7, 14490–14499

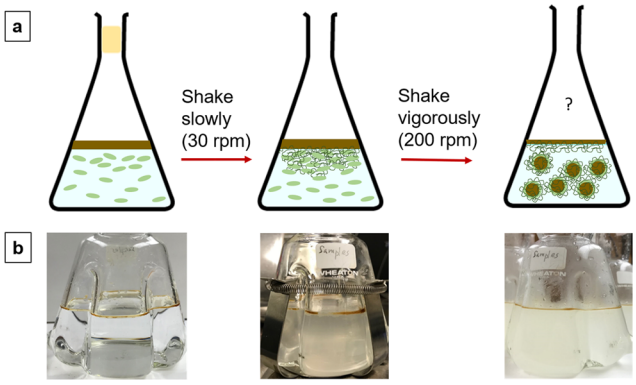


Figure 4. (a) Schematic of the experiment designed for simulating dispersion of a biofilm coated oil slick, (b) digital photos of the baffled flask corresponding to the schematic, from left to right: immediately after introduction of the inoculum, after incubation for 3 days, and after shaking the 3-day system and allowing it to settle following the baffled flask test protocol.

The results of the baffled flask test are presented in Figure 5. The freshly introduced inoculum has a minimal effect on oil

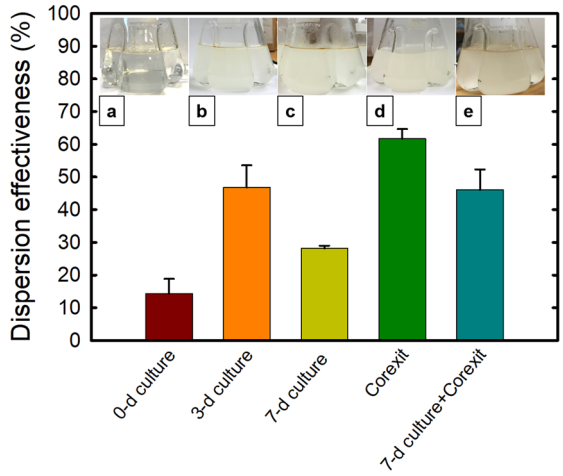


Figure 5. Anadarko crude oil dispersed by *A. borkumensis* culture after the introduction of the inoculum (0 day culture), 3 days, and 7 days later. Dispersant to oil ratio (DOR) of Corexit is 1:100. Oil to ONR7a ratio 1:1000.

dispersion with less than 15% oil dispersed which is only slightly more than that of the natural dispersion based on small amounts of amphiphilic hydrocarbons in the crude (Figure 5, system a). The 3-day bacterial culture that contains biofilm shows a moderate dispersion of crude oil (46%) as estimated from the baffled flask test (system b). The sample containing bacterial culture incubated for 7 days, however, is less effective in oil dispersion, as only approximately 30% of oil is dispersed (system c). Not surprisingly, the bacterial culture although moderately dispersing oil, does not disperse it to the same degree as the chemical surfactant blend Corexit (system d) which when added at a 1:100 v/v ratio to the crude oil yields a dispersion of about 65%. This is the only system that does not show any distinct oil as a bulk phase over the water phase.

As seen in the samples after 3 and 7 days of incubation which contain biosurfactant, the presence of biosurfactant is not the sole factor that determines the dispersion effectiveness. The highest dispersion effectiveness with the bacterial culture was achieved with bacteria incubated for 3 days. We therefore propose that biofilm at the oil–water interface of a surface slick plays a role in the dispersibility of oil that is aged for a relatively short period of greater than perhaps 2 days, and that is separate from weathering where the lighter components of the oil evaporate leaving behind the heavier more viscous components. The process starts with cells attaching to the oil–water interface as has been noted by other researchers.[54,55] The subsequent rapid growth of biofilm[56,57] leads to interfacial rigidity and eventually inhibits the ability of any biosurfactant produced to significantly enhance the dispersibility of oil. While the role of the biosurfactant to enhance dispersion is perhaps valid for a period of up to 3 days, by day 7 the biofilm rigidity reduces the dispersibility significantly. Thus, a 3-day culture performs better in a baffled flask test compared to the 7-day culture, due to a less rigid biofilm at the interface. The visual comparison of the two samples showing the most dispersion (the 3-day culture and Corexit) is seen in the digital photographs of Figure 5. We note that the dispersion effectiveness of Corexit added to oil that is not colonized by bacteria is much higher since Corexit decreases the oil–water interfacial tension to values as low as 0.1 mN/m at a 1:100 dispersant-to-oil ratio (DOR),[58] 2 orders of magnitude lower than the lowest interfacial tension (23 mN/m) generated by the biosurfactant (Figure 1 inset).

A revealing experiment that emphasizes how a mature biofilm at the interface may hinder extensive dispersion of oil is the addition of Corexit to the 7-day culture (Figure 5e). There is a clear decrease in the dispersion effectiveness to about 50%. This is an observation that is typically not recognized in the use of chemical dispersants, where it is generally assumed that dispersion effectiveness decreases with aging because of oil weathering.[50] Our results in a system where weathering is minimal, indicate that the decrease in dispersion effectiveness is a consequence of interfacial rigidity created by the formation of a bacterial biofilm. The biofilm in the sample incubated for 7 days creates a barrier at the interface hindering breakage of the slick into smaller droplets through agitation. It should be noted that the increased rigidity of the biofilm may be the result of the changes in the bacterial culture, specifically, due to the increased number of dead bacterial cells as can be deduced from the growth curve on Figure 1. The growth as monitored by resazurin assay reveals that the number of viable cells in the bacterial culture begins to decline after the first 2 days of incubation, whereas the response from the total biofilm when measured by absorbance at 600 nm continues to increase. This suggests that the presence of a mature biofilm containing dead cells affects the dispersibility of oil significantly differently as compared to a biofilm formed during an active growth phase with a lot of live cells in the system. The finding emphasizes the importance of the window-of-opportunity for dispersant applications on surface slicks, which are sensitive to oil weathering.[50] Our work suggests that microbial processes occurring at the oil–water interface of an oil slick may be an additional factor to consider.

Aliquots from the aqueous subphase were sampled for analysis by optical microscopy after the baffled flask test was done. The droplet size distribution (DSD) of the sample before incubation with only a small number of cells expectedly contains predominantly large droplets (Figure 6a) with the median droplet diameter of 16 μm and over 80% of the droplets are in the 10–20 μm range. Most of the oil does not form droplets and rises to the surface reconstituting the

DOI: 10.1021/acssuschemeng.9b01923
ACS Sustainable Chem. Eng. 2019, 7, 14490–14499

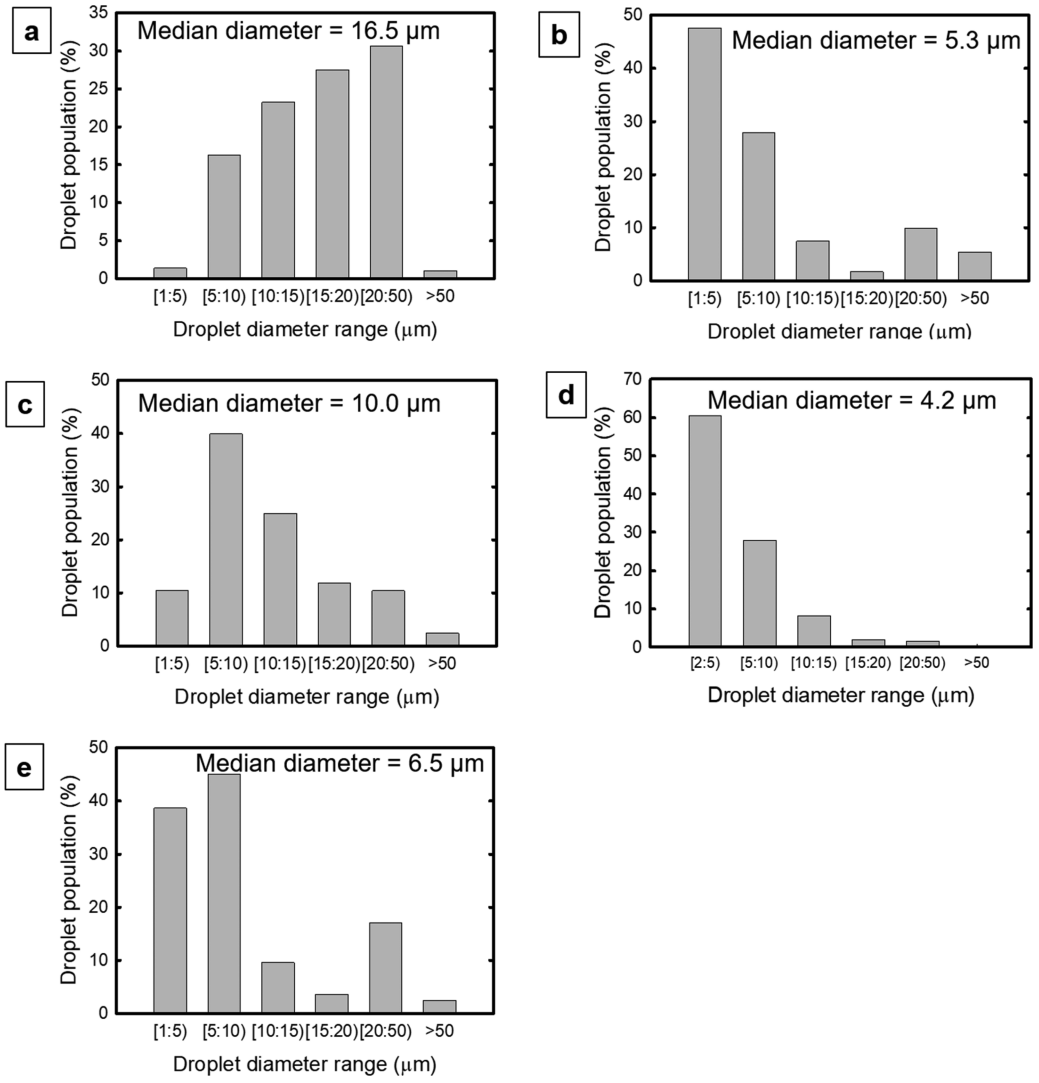


Figure 6. Droplet size distributions in the subphase of the baffled flask reflecting (a) dispersion in an *A. borkumensis* inoculum immediately after introduction, (b) dispersion in a 3-day *A. borkumensis* culture, (c) dispersion in a 7-day *A. borkumensis* culture, (d) dispersion by Corexit at DOR 1:100, (e) dispersion by Corexit at DOR 1:100 added to the bacterial sample incubated for 7 days. The Anadarko crude oil to ONR7a ratio is 1:1000 in all experiments.

initially present oil slick, as can also be seen from the flask images in Figures 4a and 5a. As the bacterial density increases in the 3-day culture (Figure 6b), smaller droplets were formed with the generation of biosurfactant. Almost half of the analyzed droplets in the 3-day cultured sample of Figure 5b are in the 1−5 μm range (Figure 6b). Figure 6c shows the droplet size distribution of the 7-day cultured sample (Figure 5c). In this case, there is a median diameter of 10 μm and a decrease in the number density of droplets in the 1−5 μm range. With the control sample of Corexit, over 60% of the droplet population falls into the 1−5 μm range (Figure 6d). When oil colonized by bacteria for 7 days is dispersed with Corexit at a 1:100 DOR (Figure 6e), over half the droplets are larger than 5 μm. The smallest droplets constituted approximately 40% of the droplet population. The optical micrographs of the emulsion droplets are reported in the Supporting Information (Figure S3).

A trend corresponding to the dispersion effectiveness is observed from the DSDs in Figure 6, where the Corexit-dispersed and the 3-day culture dispersed samples contain the largest fraction of small oil droplets (1−5 μm range) (Figure 6b,d). The median droplet diameter (4−5 μm) is also comparable in the samples dispersed with the 3-day culture and with Corexit (Figure 6b,d). The droplets in both these samples are sufficiently small to render them stable in the water column indicating that oil can be effectively dispersed in both systems. The bacterially dispersed sample with a mature biofilm incubated for 7 days, however, contains larger droplets in the aqueous subphase, with a median diameter of 10 μm (Figure 6c). Together with the fact that the 7-day system shows a significantly reduced dispersion effectiveness of 30%, the observation indicates the biofilm induced rigidity of the oil−water interface and the difficulty of dispersing the oil. We note that the biosurfactant generated can reduce the interfacial tension to around 23 mN/m over 5−7 days for a pristine oil−water interface, but the dispersion effectiveness in the real system with a biofilm containing interface does not correlate to the reduction in interfacial tension. Dispersion therefore is not simply a consequence of a reduction in interfacial tension. The rigidity of the biofilm hinders formation of very small droplets even with the application of Corexit, resulting in the shift in the

DOI: 10.1021/acssuschemeng.9b01923
ACS Sustainable Chem. Eng. 2019, 7, 14490−14499

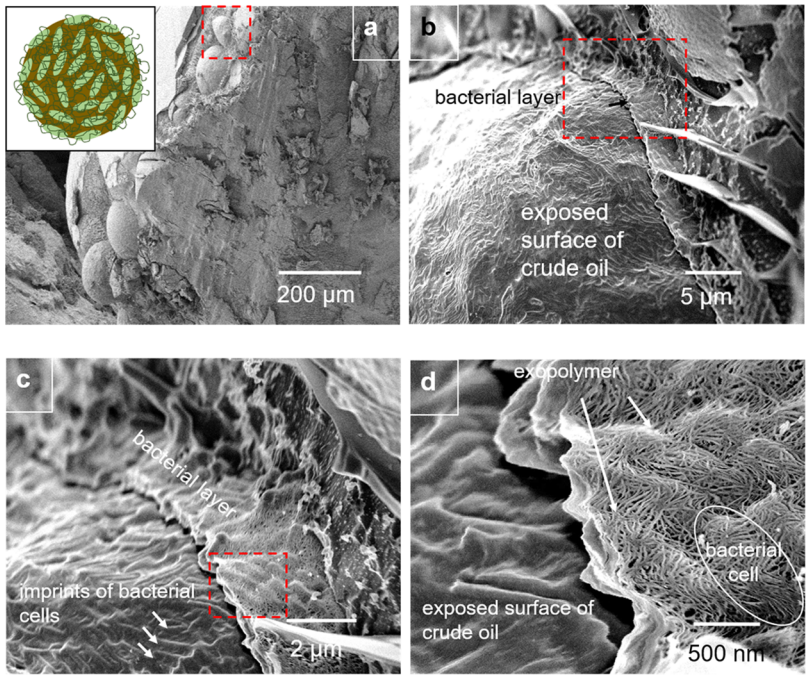


Figure 7. Cryo-SEM images of *A. borkumensis* stabilized emulsion: (a) *A. borkumensis* stabilized emulsion sample shows the presence of several droplets, (b) bacteria and exopolymer covered oil droplet has some of the adsorbed cells attached to oil, most of the surface layer was removed as a result of sample fracture, (c) higher magnification used to show the surface layer on the oil droplet, (d) high resolution cryo-SEM shows rod shaped cells typical of *A. borkumensis* morphology at the surface of oil and exopolymer interconnected the cells.

DSD toward larger diameters (Figure 6e) and a decrease in dispersion effectiveness (Figure 5e).

Cell Sequestration in Biofilms at the Droplet Oil–Water Interface. Figure 7 shows cryo-SEM images of droplets sampled from a flask incubated for 5 days and shaken to obtain an oil–water emulsion. Figure 7a is a low magnification image showing several drops from a frozen aliquot. The schematic in the inset represents the expected droplet stabilization mechanism for the bacterially dispersed samples. Figure 7b focuses on a droplet with some of the bacterial surface layer intact and most of the oil phase exposed due to the fracturing of the sample to show a surface with droplets embedded. With a focus on the bacterial layer, higher resolution images indicate that the layer is made up of rod-shaped bacteria tightly packed and with their long-axis side attached to the oil (Figure 7c). Such attachment of bacteria was suggested to occur due to potential bridging of the biosurfactant at the interface enabling bacterial attachment to the oil–water interface.[39,54] The surface of the exposed oil shows imprints of bacterial cells, also visible on Figure 7b. Figure 7d is a high-resolution image showing bacterial cells more clearly, and the exopolymer fibers are fully discernible. The exopolymer covers the surface of cells and the dense exopolymer and cells constitute the biofilm matrix.[10]

Implications to Oil Biodegradation. To complete studies on the attachment of hydrocarbon degrading bacteria, it becomes necessary to get a quantitative estimation of biodegradation. Since we are using a single bacterium cultured to degrade hexadecane, a simple test was devised to estimate crude oil biodegradation based on the consumption of hexadecane that was intentionally spiked into the oil prior to the experiment. It should be noted that hexadecane is a small component of the crude and is difficult to quantify because of the large number of components of the oil. Spiking its concentration provides clarity in peak area estimation. In analyzing characteristics of biodegradation, we used anthracene as an initial standard and also spiked the crude oil with anthracene for clarity of the GC peak. Aromatics are recalcitrant to degradation by *A. borkumensis*[59] and in the single microbial species based degradation used in this work, the added anthracene serves as a clear internal standard for understanding degradation of hexadecane. The ratio of the hexadecane peak to the anthracene peak is used as a measure of biodegradation.[38] This method facilitates solubilization of both components in crude oil, as hexadecane and anthracene are mutually insoluble but each component is soluble in the crude. The GC traces in the insets to Figure 8 show the characteristics of the hexadecane degradation using anthracene

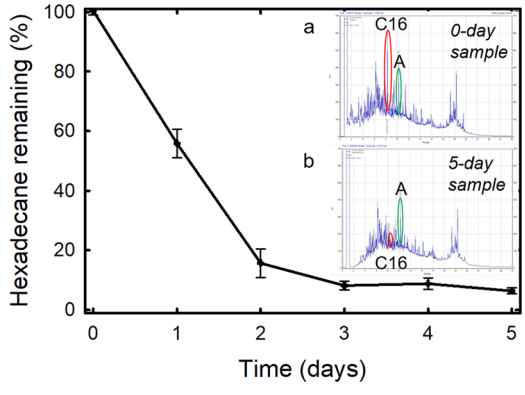


Figure 8. Biodegradation of hexadecane by *A. borkumensis*. Hexadecane in the crude oil serves as a biodegradation indicator (inset a). The error bars indicate standard error. Approximately 6% of hexadecane remained after 5 days of incubation (inset b). C16 denotes hexadecane peak, and A indicates the anthracene peak.

DOI: 10.1021/acssuschemeng.9b01923
ACS Sustainable Chem. Eng. 2019, 7, 14490–14499

as the standard. As Figure 8 indicates, hexadecane degradation in the emulsion system stabilized by *A. borkumensis* begins to proceed rapidly within the first 2 days while the anthracene is unaffected. Upon encountering an oil mixture, hydrocarbon degrading bacteria quickly adapt to the consumption of the available substrate suitable for their metabolism,[50] which explains the rapid degradation of hexadecane that *A. borkumensis* are predisposed to consume. The observation suggests that bacteria consume hexadecane at early stages of their growth before building an extensive biofilm network clearly visible at the interface. Approximately 6% of hexadecane remained after 5 days of incubation. The values are normalized against abiotic controls which showed no degradation of hexadecane over the duration of the experiment. The pseudo-first-order rate constant of the hexadecane consumption is 0.74 ± 0.06 d^{-1} with a half-life of 0.94 days.

CONCLUSIONS

We have examined the emulsion stabilization capabilities of *A. borkumensis* bacterial biofilm as a natural and green method of oil droplet stabilization in seawater. The results of the work show that with sufficient simulated wave action the *A. borkumensis* bacterial culture is effective at dispersing approximately 45% of oil slick into stable droplets 10−50 μm in diameter. Cryo-SEM reveals direct adhesion of bacterial cells and exopolymer at the oil surface, indicating that bacteria and exopolymer contribute to the Pickering-type stabilization of the oil-in-water emulsion. The secreted biosurfactant additionally facilitates the formation of smaller droplets for effective dispersion of oil in the water column.

The role of exopolymer in the *A. borkumensis* biofilm was found to be especially significant in the context of oil spill dispersion. The exopolymer coats the bacterial cells as well as immobilizes them to form a rigid three-dimensional biofilm framework. A mature biofilm grown at the interface can provide resistance to dispersion as it tends to retain the shape of the interface due to the strong attachment of the cells and their self-locking with the produced exopolymer. The baffled flask test of dispersant action when added to the experimentally simulated oil slick with bacterial biofilm indicates that dispersion may be inhibited when sufficient biofilm forms that leads to a biofilm mat that rigidifies the oil−water interface. The work therefore shows characteristics of the emulsion stabilization mechanism of bacterial cultures and complements earlier work related to the understanding of the effect of surfactants on the bacterial growth and attachment. Our work suggests that in addition to the effects of weathering of spilled oil due to photooxidation and evaporation, oil spill response mechanisms may need to consider the effect of microorganism induced changes at oil−water interfaces.

We note that a recent report from the National Academies[50] provides a wealth of information on the current status of the use of dispersants and points the way to new technologies. A specific technology that has potential is the use of particle stabilized emulsions (Pickering emulsions). Our work adds to these directions as we show that Pickering emulsions can be formed with biofilm generated by oil degrading bacteria and that the biofilm accumulates at the oil−water interface. Furthermore, the bacteria generate surfactants that reduce the oil−water interfacial tension and enable dispersion. Our findings therefore lead to an increased understanding of the oil−water interface and the dispersibility of surface slicks with underlying exopolymer. The use of cryogenic electron microscopy to image the oil−water interface at nanoscale resolution provides insight into the characteristics of biofilm growth, cell sequestration, and anchoring to the interface, and is a specific novelty of our work.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssuschemeng.9b01923.

Optical images of bacterial biofilm at the oil−water interface in the capillary used for estimation the thickness of the biofilm layer; optical micrographs of the droplets taken from the subphase after the baffled flask tests used to generate droplet size distributions (DSDs) presented in Figure 6 (PDF)

Time-lapse video of bacterial movement at the crude oil−water interface (AVI)

AUTHOR INFORMATION

Corresponding Author

*Tel.: 504-865-5883. E-mail: vj@tulane.edu.

ORCID

Diane A. Blake: 0000-0002-7532-1541
Vijay John: 0000-0001-5426-7585
Alon McCormick: 0000-0002-8885-1330
Geoffrey D. Bothun: 0000-0002-7513-2417
Srinivasa R. Raghavan: 0000-0003-0710-9845
Arijit Bose: 0000-0002-2309-5087

Notes

The authors declare no competing financial interest.

The dataset for this work can be accessed through the Gulf of Mexico Research Initiative Information and Data Cooperative (GRIIDC): https://data.gulfresearchinitiative.org, DOI: 10.7266/n7-xqtg-jd07.

ACKNOWLEDGMENTS

Funding from the Gulf of Mexico Research Initiative is gratefully acknowledged.

REFERENCES

(1) Flemming, H. C.; Wingender, J. The biofilm matrix. *Nat. Rev. Microbiol.* **2010**, *8* (9), 623−633.

(2) Flemming, H. C.; Neu, T. R.; Wozniak, D. J. The EPS matrix: The "House of Biofilm cells". *J. Bacteriol.* **2007**, *189* (22), 7945−7947.

(3) Decho, A. W.; Gutierrez, T. Microbial Extracellular Polymeric Substances (EPSs) in Ocean Systems. *Front. Microbiol.* **2017**, *8*, 922 DOI: 10.3389/fmicb.2017.00922.

(4) Tian, Y. Behaviour of bacterial extracellular polymeric substances from activated sludge: a review. *Int. J. Environ. Pollut.* **2008**, *32* (1), 78−89.

(5) More, T. T.; Yadav, J. S. S.; Yan, S.; Tyagi, R. D.; Surampalli, R. Y. Extracellular polymeric substances of bacteria and their potential environmental applications. *J. Environ. Manage.* **2014**, *144*, 1−25.

(6) Sutherland, I. W. The biofilm matrix - an immobilized but dynamic microbial environment. *Trends Microbiol.* **2001**, *9* (5), 222−227.

(7) Czaczyk, K.; Myszka, K. Biosynthesis of extracellular polymeric substances (EPS) and its role in microbial biofilm formation. *Pol J. Environ. Stud* **2007**, *16* (6), 799−806.

(8) Pal, A.; Paul, A. K. Microbial extracellular polymeric substances: central elements in heavy metal bioremediation. *Indian J. Microbiol.* **2008**, *48* (1), 49−64.
(9) Freeman, C.; Lock, M. A. The Biofilm Polysaccharide Matrix - a Buffer against Changing Organic Substrate Supply. *Limnol. Oceanogr.* **1995**, *40* (2), 273−278.
(10) Xiao, J.; Klein, M. I.; Falsetta, M. L.; Lu, B. W.; Delahunty, C. M.; Yates, J. R.; Heydorn, A.; Koo, H. The Exopolysaccharide Matrix Modulates the Interaction between 3D Architecture and Virulence of a Mixed-Species Oral Biofilm. *PLoS Pathog.* **2012**, *8* (4), e1002623.
(11) Costerton, J. W.; Cheng, K. J.; Geesey, G. G.; Ladd, T. I.; Nickel, J. C.; Dasgupta, M.; Marrie, T. J. Bacterial Biofilms in Nature and Disease. *Annu. Rev. Microbiol.* **1987**, *41*, 435−464.
(12) Hobley, L.; Harkins, C.; MacPhee, C. E.; Stanley-Wall, N. R. Giving structure to the biofilm matrix: an overview of individual strategies and emerging common themes. *Fems Microbiol Rev.* **2015**, *39* (5), 649−669.
(13) Henrici, A. T. Studies of Freshwater Bacteria: I. A Direct Microscopic Technique. *J. Bacteriol.* **1933**, *25* (3), 277−287.
(14) Bixler, G. D.; Bhushan, B. Biofouling: lessons from nature. *Philos. Trans. R. Soc., A* **2012**, *370* (1967), 2381−2417.
(15) Subramanian, S. B.; Yan, S.; Tyagi, R. D.; Surampalli, R. Y. Extracellular polymeric substances (EPS) producing bacterial strains of municipal wastewater sludge: Isolation, molecular identification, EPS characterization and performance for sludge settling and dewatering. *Water Res.* **2010**, *44* (7), 2253−2266.
(16) Guibaud, G.; van Hullebusch, E.; Bordas, F.; d'Abzac, P.; Joussein, E. Sorption of Cd(II) and Pb(II) by exopolymeric substances (EPS) extracted from activated sludges and pure bacterial strains: Modeling of the metal/ligand ratio effect and role of the mineral fraction. *Bioresour. Technol.* **2009**, *100* (12), 2959−2968.
(17) Bhaskar, P. V.; Bhosle, N. B. Bacterial extracellular polymeric substance (EPS): A carrier of heavy metals in the marine food-chain. *Environ. Int.* **2006**, *32* (2), 191−198.
(18) Decho, A. W. Microbial Exopolymer Secretions in Ocean Environments - Their Role(S) in Food Webs and Marine Processes. *Oceanogr Mar Biol.* **1990**, *28*, 73−153.
(19) Dobretsov, S.; Abed, R. M. M.; Teplitski, M. Mini-review: Inhibition of biofouling by marine microorganisms. *Biofouling* **2013**, *29* (4), 423−441.
(20) Hazen, T. C.; Prince, R. C.; Mahmoudi, N. Marine Oil Biodegradation. *Environ. Sci. Technol.* **2016**, *50* (5), 2121−2129.
(21) Shavandi, M.; Mohebali, G.; Haddadi, A.; Shakarami, H.; Nuhi, A. Emulsification potential of a newly isolated biosurfactant-producing bacterium, Rhodococcus sp. strain TA6. *Colloids Surf., B* **2011**, *82* (2), 477−482.
(22) Cameotra, S. S.; Bollag, J. M. Biosurfactant-enhanced bioremediation of polycyclic aromatic hydrocarbons. *Crit. Rev. Environ. Sci. Technol.* **2003**, *33* (2), 111−126.
(23) Wolfe, D. A.; Hameedi, M. J.; Galt, J. A.; Watabayashi, G.; Short, J.; Oclaire, C.; Rice, S.; Michel, J.; Payne, J. R.; Braddock, J.; Hanna, S.; Sale, D. The Fate of the Oil Spilled from the Exxon-Valdez. *Environ. Sci. Technol.* **1994**, *28* (13), A560−A568.
(24) Gill, D. A.; Ritchie, L. A.; Picou, J. S.; Langhinrichsen-Rohling, J.; Long, M. A.; Shenesey, J. W. The Exxon and BP oil spills: a comparison of psychosocial impacts. *Nat. Hazards* **2014**, *74* (3), 1911−1932.
(25) Yin, L. P.; Zhang, M.; Zhang, Y. L.; Qiao, F. L. The long-term prediction of the oil-contaminated water from the Sanchi collision in the East China Sea. *Acta Oceanol. Sin.* **2018**, *37* (3), 69−72.
(26) Broje, V.; Keller, A. A. Improved mechanical oil spill recovery using an optimized geometry for the skimmer surface. *Environ. Sci. Technol.* **2006**, *40* (24), 7914−7918.
(27) Mullin, J. V.; Champ, M. A. Introduction/overview to in situ burning of oil spills. *Spill Sci. Technol. Bull.* **2003**, *8* (4), 323−330.
(28) Prince, R. C. Oil Spill Dispersants: Boon or Bane? *Environ. Sci. Technol.* **2015**, *49* (11), 6376−6384.
(29) Kleindienst, S.; Seidel, M.; Ziervogel, K.; Grim, S.; Loftis, K.; Harrison, S.; Malkin, S. Y.; Perkins, M. J.; Field, J.; Sogin, M. L.; Dittmar, T.; Passow, U.; Medeiros, P. M.; Joye, S. B. Chemical dispersants can suppress the activity of natural oil-degrading microorganisms. *Proc. Natl. Acad. Sci. U. S. A.* **2015**, *112* (48), 14900−14905.
(30) Pickering, S. U. CXCVI.—Emulsions. *J. Chem. Soc., Trans.* **1907**, *91* (0), 2001−2021.
(31) Gong, H. Y.; Bao, M. T.; Pi, G. L.; Li, Y. M.; Wang, A. Q.; Wang, Z. N. Dodecanol-Modified Petroleum Hydrocarbon Degrading Bacteria for Oil Spill Remediation: Double Effect on Dispersion and Degradation. *ACS Sustainable Chem. Eng.* **2016**, *4* (1), 169−176.
(32) Marti, M. E.; Colonna, W. J.; Patra, P.; Zhang, H.; Green, C.; Reznik, G.; Pynn, M.; Jarrell, K.; Nyman, J. A.; Somasundaran, P.; Glatz, C. E.; Lamsal, B. P. Production and characterization of microbial biosurfactants for potential use in oil-spill remediation. *Enzyme Microb. Technol.* **2014**, *55*, 31−39.
(33) Ma, K. Y.; Sun, M. Y.; Dong, W.; He, C. Q.; Chen, F. L.; Ma, Y. L. Effects of nutrition optimization strategy on rhamnolipid production in a Pseudomonas aeruginosa strain DN1 for bioremediation of crude oil. *Biocatal. Agric. Biotechnol.* **2016**, *6*, 144−151.
(34) Liu, G. S.; Zhong, H.; Yang, X.; Liu, Y.; Shao, B. B.; Liu, Z. F. Advances in applications of rhamnolipids biosurfactant in environmental remediation: A review. *Biotechnol. Bioeng.* **2018**, *115* (4), 796−814.
(35) Schwehr, K. A.; Xu, C.; Chiu, M. H.; Zhang, S. J.; Sun, L. N.; Lin, P.; Beaver, M.; Jackson, C.; Agueda, O.; Bergen, C.; Chin, W. C.; Quigg, A.; Santschi, P. H. Protein: Polysaccharide ratio in exopolymeric substances controlling the surface tension of seawater in the presence or absence of surrogate Macondo oil with and without Corexit. *Mar. Chem.* **2018**, *206*, 84−92.
(36) Yakimov, M. M.; Golyshin, P. N.; Lang, S.; Moore, E. R. B.; Abraham, W. R.; Lunsdorf, H.; Timmis, K. N. Alcanivorax borkumensis gen. nov., sp. nov., a new, hydrocarbon-degrading and surfactant-producing marine bacterium. *Int. J. Syst. Bacteriol.* **1998**, *48*, 339−348.
(37) Abraham, W. R.; Meyer, H.; Yakimov, M. Novel glycine containing glucolipids from the alkane using bacterium Alcanivorax borkumensis. *Biochim. Biophys. Acta, Lipids Lipid Metab.* **1998**, *1393* (1), 57−62.
(38) Omarova, M.; Swientoniewski, L. T.; Mkam Tsengam, I. K.; Panchal, A.; Yu, T.; Blake, D. A.; Lvov, Y. M.; Zhang, D.; John, V. Engineered Clays as Sustainable Oil Dispersants in the Presence of Model Hydrocarbon Degrading Bacteria: The Role of Bacterial Sequestration and Biofilm Formation. *ACS Sustainable Chem. Eng.* **2018**, *6* (11), 14143−14153.
(39) Godfrin, M. P.; Sihlabela, M.; Bose, A.; Tripathi, A. Behavior of Marine Bacteria in Clean Environment and Oil Spill Conditions. *Langmuir* **2018**, *34* (30), 9047−9053.
(40) MacNaughton, S. J.; Swannell, R.; Daniel, F.; Bristow, L. Biodegradation of dispersed forties crude and Alaskan North Slope oils in microcosms under simulated marine conditions. *Spill Sci. Technol. Bull.* **2003**, *8* (2), 179−186.
(41) Head, I. M.; Jones, D. M.; Roling, W. F. M. Marine microorganisms make a meal of oil. *Nat. Rev. Microbiol.* **2006**, *4* (3), 173−182.
(42) Bidwell, J. P.; Spotte, S. H. *Artificial Seawaters*, 1st ed.; Jones and Bartlett Learning, 1985.
(43) *ONR7a MEDIUM*; DSMZ GmbH, 2018.
(44) Duarte, M.; Giordani, R. B.; De Carli, G. A.; Zuanazzi, J. A.; Macedo, A. J.; Tasca, T. A quantitative resazurin assay to determinate the viability of Trichomonas vaginalis and the cytotoxicity of organic solvents and surfactant agents. *Exp. Parasitol.* **2009**, *123* (2), 195−198.
(45) Srinivasan, R.; Lu, Q.; Sorial, G. A.; Venosa, A. D.; Mullin, J. Dispersant effectiveness of heavy fuel oils using the baffled flask test. *Environ. Eng. Sci.* **2007**, *24* (9), 1307−1320.
(46) Hara, A.; Syutsubo, K.; Harayama, S. Alcanivorax which prevails in oil-contaminated seawater exhibits broad substrate specificity for alkane degradation. *Environ. Microbiol.* **2003**, *5* (9), 746−753.

DOI: 10.1021/acssuschemeng.9b01923
ACS Sustainable Chem. Eng. 2019, 7, 14490−14499

(47) Bookstaver, M.; Godfrin, M. P.; Bose, A.; Tripathi, A. An insight into the growth of Alcanivorax borkumensis under different inoculation conditions. *J. Pet. Sci. Eng.* **2015**, *129*, 153−158.
(48) Scoma, A.; Barbato, M.; Borin, S.; Daffonchio, D.; Boon, N. An impaired metabolic response to hydrostatic pressure explains Alcanivorax borkumensis recorded distribution in the deep marine water column. *Sci. Rep.* **2016**, *6*, 31316 DOI: 10.1038/srep31316.
(49) Barbato, M.; Scoma, A.; Mapelli, F.; De Smet, R.; Banat, I. M.; Daffonchio, D.; Boon, N.; Borin, S. Hydrocarbonoclastic Alcanivorax Isolates Exhibit Different Physiological and Expression Responses to n-dodecane. *Front. Microbiol.* **2016**, *7*, 2056 DOI: 10.3389/fmicb.2016.02056.
(50) National Academies of Sciences; Engineering and Medicine *The Use of Dispersants in Marine Oil Spill Response*; The National Academies Press: Washington, DC, 2019.
(51) Niepa, T. H. R.; Vaccari, L.; Leheny, R. L.; Goulian, M.; Lee, D.; Stebe, K. J. Films of Bacteria at Interfaces (FBI): Remodeling of Fluid Interfaces by Pseudomonas aeruginosa. *Sci. Rep.* **2017**, *7*, 17864 DOI: 10.1038/s41598-017-17721-3.
(52) Ruhs, P. A.; Bocker, L.; Inglis, R. F.; Fischer, P. Studying bacterial hydrophobicity and biofilm formation at liquid-liquid interfaces through interfacial rheology and pendant drop tensiometry. *Colloids Surf., B* **2014**, *117*, 174−184.
(53) Klein, B.; Bouriat, P.; Goulas, P.; Grimaud, R. Behavior of Marinobacter hydrocarbonoclasticus SP17 Cells During Initiation of Biofilm Formation at the Alkane-Water Interface. *Biotechnol. Bioeng.* **2010**, *105* (3), 461−468.
(54) Abbasi, A.; Bothun, G. D.; Bose, A. Attachment of Alcanivorax borkumensis to Hexadecane-In-Artificial Sea Water Emulsion Droplets. *Langmuir* **2018**, *34* (18), 5352−5357.
(55) Panchal, A.; Swientoniewski, L. T.; Omarova, M.; Yu, T. Y.; Zhang, D. H.; Blake, D. A.; John, V.; Lvov, Y. M. Bacterial proliferation on clay nanotube Pickering emulsions for oil spill bioremediation. *Colloids Surf., B* **2018**, *164*, 27−33.
(56) Dunne, W. M. Bacterial adhesion: Seen any good biofilms lately? *Clin Microbiol Rev.* **2002**, *15* (2), 155.
(57) Palmer, J.; Flint, S.; Brooks, J. Bacterial cell attachment, the beginning of a biofilm. *J. Ind. Microbiol. Biotechnol.* **2007**, *34* (9), 577−588.
(58) Venkataraman, P.; Tang, J. J.; Frenkel, E.; McPherson, G. L.; He, J. B.; Raghavan, S. R.; Kolesnichenko, V.; Bose, A.; John, V. T. Attachment of a Hydrophobically Modified Biopolymer at the Oil-Water Interface in the Treatment of Oil Spills. *ACS Appl. Mater. Interfaces* **2013**, *5* (9), 3572−3580.
(59) Lofthus, S.; Netzer, R.; Lewin, A. S.; Heggeset, T. M. B.; Haugen, T.; Brakstad, O. G. Biodegradation of n-alkanes on oil-seawater interfaces at different temperatures and microbial communities associated with the degradation. *Biodegradation* **2018**, *29* (2), 141−157.

DOI: 10.1021/acssuschemeng.9b01923
ACS Sustainable Chem. Eng. 2019, 7, 14490−14499

Supporting information

Biofilm Formation by Hydrocarbon-Degrading Marine Bacteria and its Effects on Oil Dispersion

Marzhana Omarova[1], Lauren T. Swientoniewski[2], Igor Kevin Mkam Tsengam[1], Diane A. Blake[2], Vijay John[1], Alon McCormick[3], Geoffrey D. Bothun[4], Srinivasa R. Raghavan[5], Arijit Bose[4]*

[1]Department of Chemical and Biomolecular Engineering, Tulane University, 300 Lindy Boggs Building, New Orleans, LA 70118

[2]Tulane University School of Medicine, 1430 Tulane Avenue, New Orleans, LA 70112

[3]Department of Chemical Engineering and Material Science, University of Minnesota, 421 Washington avenue SE, Minneapolis, MN 55455

[4]Department of Chemical Engineering, University of Rhode Island, 51 Lower College Road, Kingston, RI 02881

[5]Department of Chemical and Biomolecular Engineering, University of Maryland, 4418 Stadium Drive, College Park, MD 20742

Number of pages: 3

Supporting information contains:

Video S1. Time-lapse of bacterial movement at the oil-water interface

Figure S2. Anadarko crude oil – ONR7a medium interface

Figure S3. Optical micrographs of crude oil droplets in the subphase of the baffled flask

* Corresponding author: Phone: 504-865-5883. E-mail: vj@tulane.edu.

The experimental design involved *A.borkumensis* culture and a capillary (OD=0.1mm) filled with ONR7a medium with 1 µl of crude oil injected with a thin needle. The capillary was secured onto a petri dish and the dish was filled with more ONR7a medium. Bacterial inoculum (1:100 v/v) was introduced into the petri dish containing oil and ONR7a medium filled capillary

S1. The video is a time-lapse of bacterial movement at the oil-water interface 24 hours after bacteria addition. The time-lapse is 10x speeded. The cells further from the interface appear to be moving more vigorously. The first biofilm tendrils appear at the oil surface.

S2. Immobilized crude oil – water interface in the capillary to show biofilm formation and estimate the thickness of the biofilm layer.

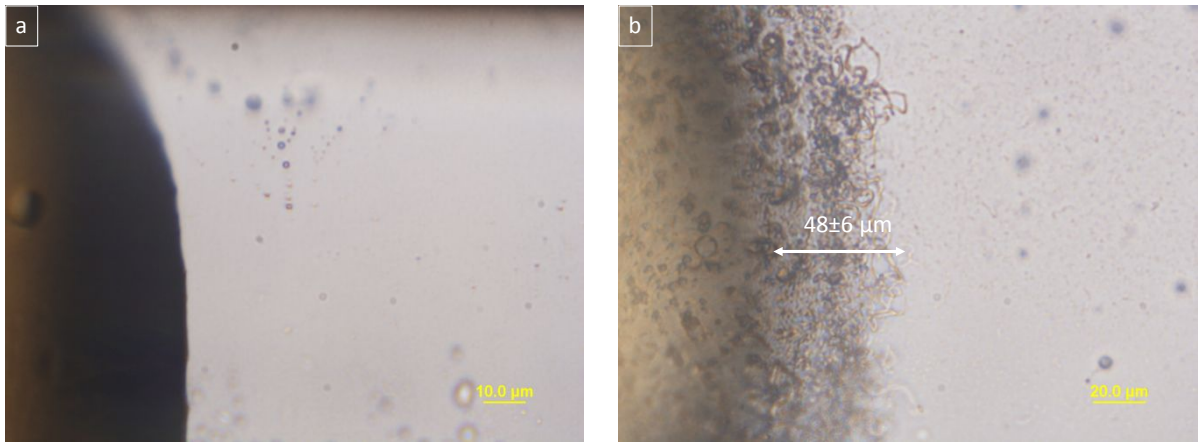


Figure S2. Anadarko crude oil – ONR7a medium interface: (a) before *A.borkumensis* biofilm growth, (b) 72 hours after the introduction of bacteria. After 72 hours a biofilm mat is observed covering the oil surface.

S3. Optical micrographs of the droplets taken from the subphase after the baffled flask tests. The images were used to generate droplet size distributions (DSDs) presented in Figure 6 of the manuscript.

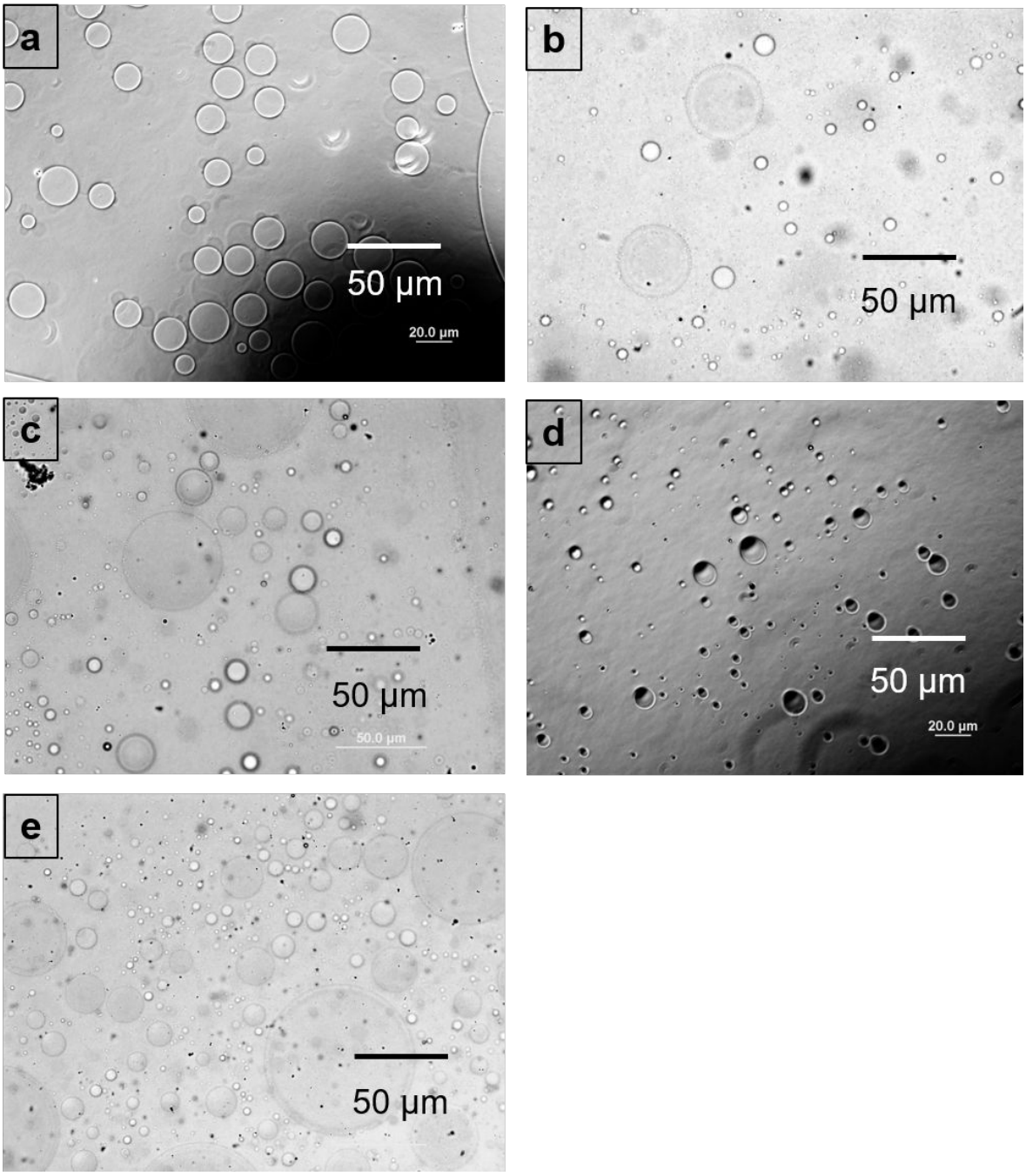


Figure S3. Droplets in the subphase of the baffled flask generated by: (a) dispersion in an *A.borkumensis* inoculum immediately after introduction, (b) dispersion in a 3-day *A.borkumensis* culture, (c) dispersion in a 7-day *A.borkumensis* culture, (d) dispersion by Corexit at DOR 1:100, (e) dispersion by Corexit at DOR 1:100 added to the bacterial sample incubated for 7 days. The Anadarko crude oil to ONR7a ratio is 1:1000 in all experiments.