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SURFACTANT ENHANCED BIOREMEDIATION OF OIL BY
ALCANIVORAX BORKUMENSIS USING FOOD GRADE
AMPHIPHILES
BY
JOSEPH ROCCHIO

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
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MASTER OF SCIENCE THESIS

OF

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ABSTRACT

The Deepwater Horizon disaster in 2010 led to the release of 200 million gallons of crude oil into the ocean, and really put into perspective the amount of damage that man can inflict on the environment. One way that first responders attempted to deal with the spill was by dispersing the oil into the water column with the use of a chemical mixture known as Corexit 9500. Being sprayed onto the slick from above, and down near the wellhead below, this mixture of surfactants and solvents was aimed at stopping the slick from reaching the coast and also speeding up the bioremediation process by increasing the specific surface area of the slick. And while Corexit has been shown in prior studies to be effective at performing this task, certain components that make up Corexit, mainly the surfactant dioctyl sodium sulfosuccinate (DOSS), have environmental concerns surrounding them including effects to human health, persistence in the ocean and possible impairment of the bacterial oil degradation. Therefore, there is a need for a more environmentally friendly dispersant.

Recently, an alternative dispersant mixture being composed of the food grade amphiphiles lecithin and Tween 80 has shown much promise in exhibiting similar dispersant effectiveness as Corexit 9500. The studies performed in this thesis investigate these mixtures further, while also trying to understand the dispersion and degradation processes.

The first manuscript investigates a solvent free mixture of lecithin and Tween 80, being composed of lecithin and Tween 80 assemblies formed in water. Investigation through Cryo TEM revealed that the combination of these two amphiphiles forms bicelles in water. Dispersant effectiveness testing showed the lecithin/Tween 80 solvent free system to be effective at forming a stable emulsion.

The second manuscript investigates the oil biodegradation process of oil with *Alcanivorax borkumensis* when supplemented with a variety of dispersant mixtures. Emphasis was given to the newly developed lecithin-Tween 80 mixture, which was compared to the controls of no dispersant and Corexit 9500. Lecithin alone and Tween 80 alone dispersant mixtures were also tested in order to elucidate whether it is the rise in bioavailability or the presence of one of the two surfactants that gives the lecithin-Tween 80 mixture its degradation behavior. Microscopic analysis of the systems showed the presence of bacteria-oil agglomerates for all but Tween 80. And oil degradation quantification results after 144 hours showed lecithin/Tween 80 to enhance degradation the most, at 52%; compared to no dispersant at 25%, Corexit 9500 at 35%, Tween 80 at 35% and lecithin at 38%.

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PREFACE

The following thesis is presented in manuscript format.

The truth may be stretched thin, but it never breaks, and it always surfaces above lies, as oil floats
on water.

Miguel de Cervantes Saavedra, *Don Quixote: Part Two*

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CHAPTER 1 : INTRODUCTION

The Deepwater Horizon Oil Spill of 2010 was a monumental event that elucidated not only the consequences that humanity's actions can wreak on the environment but also highlighted how, at times, we are ill informed to adequately address the problems we create. During the oil spill, 1.84 million gallons of a chemical mixture known as Corexit 9500A—composed of nonionic and anionic surfactants, and propylene glycol and petroleum distillates—were applied to the slick in order to promote the biodegradation of the oil through a process known as dispersion². In the act of dispersion, the surfactants reduce the interfacial tension between oil and water and thus enable the slick to be broken apart into tiny droplets. These droplets are then dispersed or “sink” into the aqueous phase, preventing the oil slick from traveling to the shore and harming coastal ecosystems. This process also results in a larger specific surface area for the oil which has been shown to increase the rate of remediation by hydrocarbon degrading microbes.³

This thesis investigates the processes of dispersant mixtures in regards to dispersant effectiveness and also degradation using a bacterial culture of *Alcanivorax borkumensis* at the benchtop scale. Emphasis is given to a recently developed dispersant mixture using food grade amphiphiles, which has been shown to exhibit similar dispersant effectiveness to Corexit 9500.⁴

Chapter 2 gives a background of the material investigated in this thesis. It is split into seven sections and should be read for more in depth knowledge regarding the studies performed in the subsequent manuscripts.

Chapter 3 is the first manuscript, “A solvent free lecithin-Tween 80 mixture for oil dispersion.” This study has been submitted and accepted to Colloids and Surfaces A: Physicochemical and Engineering Aspects.

Chapter 4 is the second manuscript, “Surfactant enhanced bioremediation of oil by *Alcanivorax borkumensis* using food grade amphiphiles.” This study is still in preparation, to be submitted to Marine Pollution Bulletin.

CHAPTER 2 : BACKGROUND

1. Dispersant usage

While the prevalence of oil spills has been trending downward in recent years as technologies improve and prevention plans are put into place, even one oil spill poses an enormous threat to ecosystems and marine life in the nearby environment.⁵ This fact was particularly brought to light by the Deepwater Horizon disaster in 2010, where an explosion led to the discharge of about 200 million gallons of crude oil into the Gulf of Mexico⁶, an event which left an entire ecosystem devastated⁷.

During the spill in 2010, a variety of methods were employed in order to combat the spill and therefore mitigate any damage that the oil may cause to the marine environment. These methods included in-situ burning, physical removal with skimmers, containment of oil with booms, and the usage of a chemical mixture known as a dispersant. (Figure 2.1 shows a variety of methods both man-made and natural that control the oil's ultimate fate)

Figure 2.1 Various methods of oil cleanup.⁸

Out of the methods used, the one that the present thesis is interested in studying is the usage of dispersants. Compared to the other methods, dispersant application has been shown to be the most effective while costing the least amount of man power and money. A review by Lessard lists the reasons why dispersant usage is growing in popularity and chosen more and more as the cleanup method of choice: it can be used in extreme weather conditions (high winds and large waves even raising its efficacy), the use of airplanes to spread the dispersant can be done very quickly (seen in Figure 2.2), it limits the formation of water in oil emulsions known as “chocolate mousse” which prove to be very problematic in cleanup, it increases the surface area and therefore bioavailability of the oil (which has been shown to increase biodegradation rate), and the dispersants themselves are made up of largely benign, even biodegradable components.³

Figure 2.2: The application of dispersants from the air and subsequent physical process¹

A dispersant is made up of two types of molecules: surfactants, amphiphilic molecules that due to their hydrophobic and hydrophilic components can pack themselves at the oil/water interface and lower the interfacial tension, which thus enables the formation of a dispersion with less energy; and the solvent, which is the “carrier” of the surfactants and assists the surfactants in

penetrating the oil slick and reaching the interface.^{3,9} The dispersant that has seen the most use in the field is Corexit 9500, a mixture developed by Exxonmobil that contains the surfactants Span 80, Tween 80 and 85, and dioctyl sulfosuccinate sodium salt (DOSS) dissolved in propylene glycol and petroleum distillates (packing of Corexit 9500's components at an oil/water interface can be seen in Figure 2.3). The exact concentration (w/w) of the surfactants are 18% DOSS, 4.4 % Span 80, 18% Tween 80, and 4.6% Tween 85¹⁰. Span and the Tweens are both nonionic and proven to be biodegradable, while DOSS is anionic and has some concerns surrounding it that it can persist in the environment for a longer time¹¹. Due to some of the concerns surrounding DOSS and some studies showing Corexit inhibiting oil degradation, there is much interest to develop alternative dispersants: dispersants that are just as effective as Corexit but with less adverse impact on the environment and human health.

Figure 2.3: Packing of Corexit's components at an oil/water interface⁹

2. Lecithin Tween dispersant mixtures

One alternative dispersant proven to be quite effective is seen in a paper written by Athas, in which lecithin and Tween 80 are added to an ethanol solvent and shown to exhibit similar and

even in some cases better dispersion ability than Corexit.⁴ Compared to Corexit, the lecithin/Tween 80 dispersant has safer, more environmentally friendly components. The main concern with the Corexit mixture was the nonionic surfactant DOSS which is considered to be an irritant to the eyes and skin¹² and also has been shown to slow the oxidation rate of crude oil by bacteria as well as the bacterial growth rate^{13,14}. DOSS also persists longer in the environment than the other components of CXT.¹¹

The components of the LT mixtures, however, are considered “food-grade” due to the fact that they are both ingestible by humans and present in many consumable products. Tween 80 (the molecular structure of which is seen in Figure 2.4) is often a main ingredient of ice cream¹⁵, while Lecithin (Figure 2.5) is used as an emulsifier in the food industry, as liposomes for drug delivery, and can be found as a supplement in almost any pharmacy.¹⁶

Figure 2.4: Molecular structure of Tween-80 (polysorbate 80)¹⁷

R, R' = fatty acid residues

Figure 2.5: Molecular structure of lecithin¹⁸

The lecithin/Tween 80 dispersant mixture is effective due to a synergistic effect that occurs between both surfactants. The synergistic effect can be explained by three main factors. First, the ability of both surfactants to pack tightly at the interface (in part due to the kinks in the hydrocarbon tails of both components). Second, the lecithin has a very low solubility in water and therefore does not desorb into the water phase. And third, the Tween 80's large head groups stabilize the droplets by steric repulsion. This effect is shown visually in Figure 2.6.⁴

Figure 2.6: Synergistic effect between Lecithin and Tween-80 at the oil/water interface.⁴

Further investigations have been performed with LT mixtures by Riehm et al that looked at the effect of Lecithin:Tween (w/w) and surfactant:solvent (v/v) ratio on the dispersion effectiveness.¹⁹ In the experiments done, Riehm et al. used the Baffled Flask Test to quantify the effectiveness of each dispersant. This test is a standardized technique used by the United States Environmental Protection Agency when wishing to judge the quality of various dispersants.

In a study where the L:T ratio was varied, it was shown that the most effective dispersions were formed in the range between a 60% and 80% lecithin by weight mixture (see Figure 1.7). This same test also pointed out that the application of lecithin alone performed better than Tween-80 alone.

Figure 2.7: Dispersant effectiveness as a function of lecithin/Tween-80 ratio¹⁹

Another experiment performed by Riehm showed the effect of surfactant: solvent ratio on the dispersion effectiveness. The mixture of 80% surfactant gave the best results (see Figure 2.8).

Figure 2.8: The dispersant effectiveness as a function of surfactant/oil and surfactant/solvent ratio¹⁹

Clearly, the lecithin/Tween 80 dispersant mixtures show tremendous promise and warrant further investigation. The next step for them was to be tested side by side with Corexit in a biodegradation study, as our tests hope to do.

3. Hydrocarbonoclastic bacteria

All throughout the world's oceans, there exist hydrocarbonoclastic bacteria (HCB), microorganisms capable of breaking down hydrocarbons released into the environment. These bacteria have evolved due to the presence of hydrocarbon seeps, locations in the ocean where oil is naturally emitted, and because of these bacteria's ability to metabolize oil, they are therefore able to respond to any type of disaster involving the release of oil into the sea. One genus of HCB that has been shown to be ubiquitous throughout the world (see Figure 2.9) and has intrigued

scientists interested in the mechanism behind oil degradation is *Alcanivorax*, a marine Gammaproteobacteria capable of growing on linear and branched alkanes.²⁰

Figure 2.9: Locations in the world where *Alcanivorax* and *Alcanivorax*-like bacteria have been found.²⁰

Alcanivorax, a genus initially isolated on the German island of Borkum, had interested scientists so much that it even had its entire genome mapped, the first HCB to have been done so.²¹ Some important characteristics of *Alcanivorax* are that they are rod shaped, gram negative, aerobic, oxidase and catalase positive, moderately halophilic (optimum NaCl conc between 3 and 10 %), and mesophilic.²⁰ And as stated before, *Alcanivorax* is ubiquitous--having been identified in a large variety of marine environments: surface water, shallow, deep sea, hydrothermal vents, mud volcanos, and even within gray whale carcasses²²⁻²⁵. In unpolluted waters *Alcanivorax* is found in low numbers, but seen in higher quantities in oil polluted waters and coastlines (in some places it has been shown to populate up to 80-90% of microbial community)²⁶. Due to these factors, previous studies have used *Alcanivorax* as a model bacterium for the study of the oil degradation process^{14,27}. We have chosen to use *Alcanivorax* here as well.

Figure 2.10: Mechanisms of *Alcanivorax borkumensis*²⁸

Alcanivorax borkumensis, the most studied species of the *Alcanivorax* genus, is able to perform hydrocarbon catabolism aerobically (in the presence of oxygen). There do exist other bacteria that are also able to catabolize hydrocarbons anaerobically but *A. borkumensis* does not have the metabolic machinery to do so. In order to break down the oil, *A. borkumensis* has multiple systems: it possesses AlkB1 and AlkB2, two alkane hydroxylase systems, and it also has three P450 cytochromes.²¹

A. borkumensis also possesses other functions that improve its ability to degrade hydrocarbons. *A. borkumensis* is able to produce biosurfactants, for example, which can emulsify and/or pseudosolubilize an oil and thus make it more bioavailable. It has also been shown to be able to produce EPS (extracellular polymeric substances), which enhance the bacteria's ability to adhere to the oil and also make up much of the material of the bacteria's biofilm (more on EPS and biofilm in section 6 of this chapter). *A. borkumensis* has also been shown to be able to store

lipids, scavenge for nutrients, endure immense stress, and form type IV pili.²⁰ An overview of the mechanisms of *A. borkumensis* can be seen in Figures 2.10 and 2.11.

Figure 2.11: Metabolism and processes within an *Alcanivorax borkumensis* cell.²¹

4. Surfactant enhanced bioremediation and experimentation

So far, we have discussed how dispersants function and lead to a larger distribution of oil within the water column and we have also looked at how the hydrocarbonoclastic bacteria that live in the ocean have the ability to utilize hydrocarbons as a carbon source. But does the addition of surfactant have other effects on the degradation process besides increasing the “bioavailability”? And how does one go about selecting a specific surfactant to use during an oil spill scenario?

Figure 2.12 lays out many of the variables that are important in implementing a successful application of surfactant to an oil spill. As one can imagine, the three main components to

consider are: the type of oil to be degraded (represented in the figure as NAPL, which refers to Non-Aqueous phase liquid), the type of bacteria present and the surfactant(s) used to perform the dispersion.

The interaction between the surfactant and the oil will determine the performance and characteristics of the dispersion. Characteristics that could change with surfactant choice are the stability of the emulsion, the amount of oil dispersed, the droplet size, the presence/absence of micelles and the ability of these micelles to solubilize some of the oil. These characteristics will determine the bioavailability of the oil to the bacteria.²⁹

Figure 2.12: Overview of the various relationships between components in a surfactant enhanced bioremediation process³⁰

The ability of the bacteria to adhere to the oil is something else to keep in mind when evaluating the performance of a surfactant. The ability of the bacteria to adhere is an interplay of many factors, discussed in more detail in section 5 of this chapter, but is dependent on the oil to be degraded, the surfactant used, and the kind of bacteria. The interaction between microbe and surfactant is one last thing to keep in mind, as the ability of the bacteria to biodegrade the

surfactant and also the toxicity of the surfactant will be relevant to the success of the remediation process.

As stated, dispersants have the ability to increase bioavailability in two main ways: through emulsification of the oil in droplets or through pseudo-solubilization by trapping part of the oil in its micelles.³⁰ When the oil is emulsified and exhibits a very low solubility in the aqueous phase, it is important for the bacteria to adhere to the oil droplet. In these cases, surface interactions between the surfactant and bacteria become very important. Some studies have shown that surfactants such as Tween 20²⁷ has the ability to prevent bacterial adhesion, which could definitely prevent biodegradation.

In the case of surfactants which work through micellar solubilization, there are a few important things to note. Firstly, this method cannot work at concentrations lower than the critical micelle concentration due to the fact that there are no micelles present. Secondly, there have been cases where the micellar solubilization of oil leads to the trapping of the hydrocarbon inside, and thus limiting the bioavailability.³¹ There has also been one study performed using Triton X-100 in which, even though Triton was nontoxic to the culture alone, when used to solubilize/emulsify an oil, the surfactant ended up inhibiting the biodegradation due to the toxicity of the micellar solubilized oil.³⁰ Tween 80 has been shown to raise bioavailability through micellar solubilization, but unlike Triton X-100 in the prior instance, has been shown to enhance degradation with increasing surfactant concentration above the CMC.³² An overview of the various mechanisms of oil uptake can be seen in Figure 2.13.

Figure 2.13: Mechanisms of uptake by an oil degrading bacteria

Some recent experiments performed by the Tripathi group have also answered some interesting questions regarding oil degrading bacteria and their interactions with surfactant dispersed oil. They have shown quite conclusively, that in cultures of the oil degrading bacterium *Alcanivorax borkumensis*, surface area does in fact increase the growth of the bacteria.³³ They have also shown that the surfactant Tween is degradable by these cultures and that dispersants have a large variety of ways they can affect bacterial growth.¹⁴

Scientists have also tried to elucidate the effect of dispersants on microcosms, or in other words, mixtures of a large consortia of bacterial species (usually collected from a geographic location of note). In these experiments, there has been some contradictory results as well, with some results showing an enhancement in degradation after dispersant application while others have shown an inhibition. Even through these contradictions, however, there is quite an amount of information to be gained from these experiments. Kleindienst was able to show how

dispersant-degrading bacteria could outcompete oil-degrading bacteria for nutrients and thus slow down the oil degradation.³⁴

5. Microbial attachment and droplet aggregation

As noted previously, microbial attachment to the oil can prove vital in an oil degradation scenario and therefore should be considered when analyzing the performance of a certain surfactant. One method in which scientists have attempted to interpret microbe attachment behavior is through the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory, a theory which illustrates colloidal stability/ aggregation behavior as a balance between the competing van der Waals attraction and electrostatic repulsion.³⁵

Figure 2.14 displays a separation distance vs interaction energy plot. In it you can see the contributions of the interaction energy by the individual forces: van der Waals attraction increasing as the separation distance gets smaller, as does the electrostatic and Born repulsions (Born repulsion starting only at a very small distance and then exponentially increasing). In the figure the individual contributions are denoted with the thin black lines, but the sum of the forces is the thicker black line in the middle.

An important characteristic of this graph is the energy barrier that exists in the middle between the primary and secondary minimums. The existence of this energy barrier will prevent a colloid from irreversibly aggregating, and it shows that the colloid is stable. If the energy barrier does not exist, this means that van der Waals force dominate over the electrostatic and the particles will equilibrate themselves into the low energy point of the primary minimum, thus forming an irreversible attachment/ aggregation.

Figure 2.14: Modeling of interaction energy using the relationship between electrostatic, van der Waals and Born forces³⁶

The application of DLVO theory, a model that refers to the stability and aggregation behavior of inert colloids, to microbial attachment and droplet aggregation has been used to try and predict the behavior of microorganisms in a variety of circumstances with a somewhat uneven track record due to the fact that there are some intermolecular forces not accounted for in it.³⁵ Especially with bacteria, which has been said to be an active colloid capable of biosynthesis, there are many factors that go into the behavior of the system besides just the interplay between van der Waals and electrostatic forces.

Some of these other factors (laid out in full on Figure 2.15) include the hydrophobic effect, which refers to the fact that hydrophobic entities tend to come together and have a force attached. When thinking of a system made up of bacteria and oil droplets, the hydrophobicity shows itself in the hydrophobicity of the oil and also of the cell membrane, both of which have been shown to be important in determining adhesion.³⁷ Cell surface hydrophobicity has been known to vary in

regards to different species and has also been shown to be dependent on the carbon source³⁸ and any type of environmental stress (such as the addition of a surfactant).³⁹ Bacteria may change their hydrophobicity as a survival mechanism. It has been shown in prior studies that hydrophobic cells were able to attach to flocs much better than hydrophilic ones.⁴⁰

Steric forces have also played a role in regards to the adhesion of bacteria to interfaces. Steric refers to the effect that molecular structure has on intermolecular forces. This effect can be seen in Figure 2.14 with the function of “Born repulsion”. Born repulsion comes about due to electron clouds overlapping when molecules get too close.⁴¹ As one can imagine, this repulsive force is more prominent for larger, bulkier molecules. Tween-80, one of the surfactants shown to be a very effective emulsifier of oil when paired with Lecithin, takes advantage of its bulky head group to keep the droplets stable through steric hindrance⁴. But while this effect is a positive for the stabilization of dispersions, it could pose a problem for bacteria trying to adhere to a surface. There have been studies performed that have shown that Tween has the ability to prevent bacteria from adhering to surfaces of polystyrene⁴² and oil³⁷.

One last parameter to keep in mind in regards to the various factors that may determine bacterial adhesion is the biochemical element. The fact that the bacteria are not simply floating particles and do have a variety of mechanisms involving nutrient scavenging and salt pumps could prove to affect the ability or desire of bacteria to attach to an interface.

Figure 2.15: Various forces determining aggregation and adhesion

6. EPS production, biofilm and marine snow formation

Most bacteria live in biofilm communities on surfaces as opposed to freely floating around in the planktonic state.⁴³ And for those bacteria that live in a biofilm community, it has been shown to be the case that over 90% of the dry mass is the biofilm matrix as opposed to the microorganisms themselves.⁴⁴ This matrix is made up of a substance known as extracellular polymeric substances (EPS), a term which refers to a large variety of molecules/ biopolymers secreted by bacteria and giving structure to the biofilm. While originally thought to be made up of polysaccharides alone, and given the name extracellular polysaccharides, it was later discovered that there are a large variety of other substances that makes up a biofilm as well: including proteins, lipids and nucleic acids. Therefore, the name was changed to extracellular polymeric substances (thus preserving the EPS acronym).⁴⁴

EPS has been shown to play a large variety of roles within a bacterial biofilm, laid out by Flemming in a review on the nature of biofilm. Some roles of the EPS are: adhesion, aggregation of bacteria, retention of water, serving as a protective barrier, and acting as a nutrient source.⁴⁴

In the marine environment, it has been shown that bacteria and phytoplankton have the ability to secrete EPS such as polysaccharides and proteins that self-assemble into the aqueous phase to form larger particles and nanogels. These nanogels then combine to form larger microgels and porous networks which have the ability to stick to any particles floating around in the ocean. ⁴⁵

The agglomeration of many of these particles, biofilm, and colloidal organic carbon forms a substance known as marine snow which is found in large quantities in the ocean. ⁴⁶ One of the impacts of the Deepwater Horizon oil spill was that a large amount of carbon was placed in the ocean which all kinds of materials and species could stick to...and agglomerates of bacteria, plankton, algae, sediment, and oil were found in large quantities sinking to the ocean floor. It is speculated that anywhere from 4-31% of the oil ended up back on the seafloor through this process. ⁴⁵

Examples of these agglomerations can be seen in Figure 2.16, where the rightmost image gives an impression of the spiderweb-like/ pearls on necklaces feature that these agglomerates can have.

Figure 2.16: Formation of agglomerates: “Fibrils in colloidal organic matter (1–3 nm thickness and 100s to 1000s of nm in length) (A) and (B) imaged by Atomic Force Microscopy, after mounting on mica, with image size of 10 3 10 μm; (C) imaged by Transmission Electron Microscopy, after staining with Ru oxychloride, and mounted on hydrophilic nanoplast resin. Image bar is 500 μm. All images show the “spiderweb” fibrillar structures appearing as pearls on necklaces; fibrillar forms of colloids were only found in surface and bottom waters, but not in mid-depth waters”⁴⁵

Other studies have been performed which give an idea of how surfactants could affect the bacterial colonization process. Figure 2.17 shows *Alcanivorax borkumensis* at the interface of a stabilized oil droplet. But in the experiments performed it was shown that the biofilm was able to be found only in the absence of surfactants such as Tween 20 or Corexit 9500. Tween 20 and Corexit both inhibited the attachment of the bacteria to the interface due to what was believed to be either steric repulsion or a biochemical element.²⁷

Figure 2.17: Formation of biofilm on an oil droplet by *Alcanivorax borkumensis* after 3 days. A) Fluorescence image (Scale bar equal to 10 μm). (b) Cryo SEM of hexadecane droplet. (c) Cryo SEM of droplet showing AB attached to hexadecane. (Scale bar equal to 5 μm)²⁷

One other speculation by scientists is that any dispersant added may have the ability to disperse the EPS and therefore lead to the inhibition of biofilm/ marine oil snow formation. The fact that a large amount of MOS was formed after DWH despite the application of millions of

gallons of Corexit seems to contradict this claim but some lab experiments have shown that Corexit does have the ability to impede MOS formation.⁴⁵

7. Light scattering by bacteria and emulsions

Optical density/ absorbance of bacterial culture is a straight forward, non-sacrificial technique that is often used to determine the growth of bacteria in a sample. This technique utilizes the fact that as the bacteria grow, and more of them enter the system, the amount of light scattering rises (and so does the total absorbance of the system).⁴⁷

Emulsion properties are also studied using light scattering techniques. Dynamic light scattering (DLS), Static Light scattering, along with many others utilize the relationship between the size of a particle and its Brownian motion/ scattering intensity in order to elucidate properties such as size distribution, molecular weight, and morphology.²⁹

In the experiments performed, absorbance was used to investigate what was going on inside of the dispersion mixtures. For an emulsion, the equations that form the relationship between the emulsion properties and the absorbance are the following:

$$T = \frac{I_T}{I_{0,R}} = \exp(-\varepsilon * x)$$

$$\varepsilon = \tau + \alpha$$

$$\tau = \frac{3 * \Phi * Q_s}{4 * r}$$

$$A = 2 - \log_{10}(100 * T)$$

In the above equations, T refers to transmittance, I_T is Intensity of transmitted light, $I_{0,R}$ is intensity of light emitted, ε is the extinction coefficient, x is the emulsion path length, α is absorption coefficient, τ is turbidity, Φ is dispersed oil fraction, Q_s is scattering efficiency, and r is the droplet radius. ²⁹

A few mathematical models using these equations are found in the Appendices section of this thesis.

CHAPTER 3 : MANUSCRIPT I

A solvent free lecithin-Tween 80 system for oil dispersion

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Abstract

Phospholipids have been extensively used to disperse oil-in-water (O/W) as emulsions in the food and pharmaceutical industries. In this work a new strategy of dispersing oil and forming O/W emulsions in synthetic seawater is described using lecithin-Tween 80 (LT) self-assemblies (vesicles and disk-like micelles). LT assemblies can emulsify gasoline or crude oil with greater than 90% emulsion (or dispersion) efficiency at a total surfactant concentration that allows for complete coverage of the oil droplet interface. Comparing the experimental results with theoretical results based on geometric surfactant packing indicates that lecithin and Tween 80 monolayers pack tightly at the oil/water interface. When compared to lecithin or Tween 80 assemblies, the mixed LT assemblies provide better emulsion stability than lecithin and comparable emulsion stability to Tween 80, suggesting that steric stabilization provided by Tween 80 was the primary stabilization mechanism. Aqueous dispersants such as the LT assemblies can be prepared on demand and may prove effective for dispersing oil phases under conditions of high mixing energy.

Keywords: Oil-in-water emulsion; Lipid vesicles; Oil dispersants; Self-assembly

Introduction

Phospholipids, which are amphiphilic molecules with a hydrophilic headgroup and two hydrophobic acyl tails, are common emulsifiers in the food and pharmaceutical industries. A recent review by Pichot et al.⁴⁸ describes the fundamental aspects of phospholipid organization at oil/water interfaces, and the structure and stability of phospholipid-stabilized O/W emulsions. As emulsifiers, phospholipids offer advantages over synthetic surfactants because they are biocompatible and can be derived from natural sources. However, the majority of phospholipids used are zwitterionic, and a disadvantage of using zwitterionic amphiphiles to create O/W

emulsions is that they do not provide electrostatic or sufficient steric repulsion between oil droplets. Inter-droplet repulsion improves emulsion stability by restricting droplet coalescence.

Athas et al.⁴ and Riehm et al.¹⁹ have recently shown that mixtures of food-grade lecithin (L), with zwitterionic phosphocholines as the major components, and Tween 80 (T), a nonionic polyethoxylated sorbitan with an oleic acid tail, dissolved in ethanol were effective at dispersing crude oil in O/W emulsions. The ability to form stable O/W emulsions stemmed from the low interfacial tensions provided by the lecithin-Tween 80 mixture (< 0.1 mN/m at L:T weight ratios between 9:1 and 1:9) as the lecithin and Tween 80 tails pack closely together, and the combination of the low hydrophilic-lipophilic balance (HLB) provided by lecithin and inter-droplet steric repulsion provided by Tween 80. Nyankson et al.⁴⁹ have also shown that lecithin is effective in emulsifying (dispersing) crude oil, but it was not as effective as Tween 80. Therefore, LT mixtures are attractive candidates for creating stable O/W emulsions and may prevent phase inversion (O/W to W/O), which has been observed for emulsions prepared with lecithin or Tween 80⁵⁰. Lecithin-containing surfactant mixtures have also proven effective at creating coatings that prevent oil deposition to hydrophobic surfaces such as bird feathers⁵¹.

This work describes the use of dispersants based on LT assemblies to emulsify oil. The rationale behind using this type of dispersant is that these solutions are aqueous and do not require the use of organic solvents to solubilize surfactants, where the solvents themselves may be toxic to aquatic species⁵². A L:T molar ratio of 60:40 (36:64 w/w) was used as this composition provides low O/W interfacial tension, ~ 0.03 mN/m, and prevents crude oil droplets stabilized in synthetic seawater from coalescing⁵³.

Self-assembled phospholipid vesicles in water are known to adsorb at oil/water interfaces and reduce the oil/water interfacial tension^{54,55}. Small unilamellar vesicles spread rapidly to form a monolayer and yield small oil droplets and stable emulsions, whereas large multilamellar vesicles remain intact at the droplet interface and are not as effective at stabilizing emulsions^{56,57}. Our results show that the LT assemblies consisted of unilamellar vesicles and disk-like micelles (also referred to as bicelles), and that these structures adsorbed onto gasoline and crude oil droplets to form O/W emulsions. While an aqueous-based dispersant formulation comprised of self-assembled structures may not be feasible for surface oil slicks, which require an organic solvent to penetrate the oil slick, they may be applicable to high mixing energy conditions such as deep-sea injection at wellheads, which was used to treat the Deepwater Horizon oil spill⁵⁸.

Experimental Methods

Materials. Tween 80 and lecithin (95% L- α -phosphatidylcholine, soy) (Avanti Lipids), Pennsylvania crude oil (Baar Products, Downingtown, PA), and gasoline (87 octane) were purchased and used as received. Artificial seawater was prepared from according to the ASTM D1141-98 standard and consisted of 24.53 g/L (419.7 mM) NaCl; 5.20 g/L (54.6 mM) MgCl₂; 4.09 g/L (28.8 mM) Na₂SO₄; 1.16 g/L (10.5 mM) CaCl₂; 0.695 g/L (9.3 mM) KCl; 0.201 g/L (2.4 mM) NaHCO₃; 0.101 g/L (0.8 mM) KBr; 0.027 g/L (0.4 mM) H₃BO₃; 0.005 g/L (< 0.1 mM) SrCl₂; and 0.003 g/L (< 0.1 mM) NaF.

Dynamic Light Scattering (DLS). Hydrodynamic diameter measurements of lecithin-Tween 80 self-assemblies were conducted on a Malvern Instruments Zetasizer Nano ZS (Worcestershire, UK). For size determination, 1 mL samples were placed in 10×10×45 mm quartz cuvettes and dynamic light scattering (DLS) was performed at room temperature (RT, 20°C) at 173°

backscatter angle with 120 s equilibration time. The hydrodynamic diameter for each sample was based on 15 scans measured in triplicate. Reported hydrodynamic diameters are based on two independent samples. The shape of the self-assemblies can strongly affect DLS measurements by altering the slip plane and leading to anisotropic diffusion coefficients. Hence, the hydrodynamic diameters reflect an average value for equivalent spheres.

Cryo Transmission Electron Microscopy (Cryo-TEM). Cryo-TEM samples were prepared at 20°C using a Vitrobot (FEI Company), which is a PC-controlled robotic assembly for sample vitrification. Quantifoil grids were used with 2 μm carbon holes on 200 square mesh copper grids (Electron Microscopy Sciences, Hatfield, PA). The sample was first equilibrated within the Vitrobot at RT and 100% humidity for 30 min. After immersing the grid into the sample, it was then removed, blotted to reduce film thickness, and vitrified in liquid ethane. The sample was then transferred to liquid nitrogen for storage. Imaging was performed at -170°C in a cooled stage (model 626 DH, Gatan Inc., Pleasanton, CA) at 200 kV using a JEOL JEM-2100 TEM (Peabody, MA).

Oil Emulsification. Lecithin and Tween 80 (60:40 mole ratio or 36:64 weight ratio) were added to deionized water at a total surfactant concentration of 2.5 mM and the solution was bath sonicated for 30 min to create a vesicle/micelle solution (Figure 3.1). Oil emulsions were prepared as a function of surfactant concentration with crude oil or gasoline initially at oil to artificial seawater volume ratios of 1:4. An aliquot of the vesicle/micelle solution was added to achieve the desired surfactant concentration and emulsification was achieved by vortex mixing for 30 s at RT. The height of the oil phase was measured before mixing and approximately 1 h after mixing. The percent emulsified was calculated based on the difference between the height of

the oil layer initially, h_i , and after emulsification, h_e (% Emulsified = $[h_i - h_e]/h_i$). Stability studies were conducted where the samples were left undisturbed for 1 day and continually photographed.

Figure 3.1: Chemical structures of lecithin and Tween 80, and a solution of lecithin-Tween 80 assemblies at a 60:40 molar ratio (36:64 w/w) in deionized water at 2.5 mM (0.24 wt%) surfactant.

Results and Discussion

Lecithin is a nearly water-insoluble amphiphile that forms bilayer vesicles in water at RT (Figure 2a), while Tween 80 is more water-soluble and forms spherical micelles above the critical micelle concentration of 0.012 mM (it should be noted that higher values approaching 0.1 mM have been reported^{59,60}) (Figure 3.2b). Cryo-TEM analysis shows that, when combined to form LT assemblies, these two surfactants yielded a mixture of vesicles (white arrows; Figure 3.2c-d) and micelles (dark arrows; Figure 3.2c-d). The micelles formed were disk-like rather than spherical, where spherical micelles would be expected for a surfactant with a packing parameter, P , less

than 1/3 ($P = 0.07$ for Tween 80⁶¹; Figure 2b). Disk-like micelles have also been observed for mixtures of egg phosphocholine or lecithin and Tween 80 at a similar Tween 80 compositions^{62,63}. In these disk-like micelles the edges are stabilized by Tween 80. Tween 80 is a cone-shaped molecule capable of adopting high curvature, while lecithin is a cylindrical-shaped molecule favoring bilayer structures with low curvature. The size distributions of the vesicles (diameter) and micelles (apparent size, roughly equivalent to length) from the cryo-TEM images were nearly identical (Figure 3.2e), and the average sizes were 28.6 ± 13.4 nm and 29.0 ± 7.5 nm, respectively. The sizes of the LT assemblies determined by cryo-TEM were confirmed by DLS based on the number-weighted distribution of the hydrodynamic diameter (Figure 3.2f). The intensity-weighted hydrodynamic diameter distribution, exhibiting a peak near 70 nm, was larger as the light scattered was dominated by the large vesicles in solution.

Figure 3.2: LT assembly characterization. Cryo-TEM micrographs of a) lecithin (2.5 mM), b) Tween 80 (25 mM), and c, d) lecithin-Tween-80 (2.5 mM). White arrows denote vesicles and dark arrows denote disk-like micelles. e) Vesicle diameter and micelle length

distributions from cryo-TEM analysis of LT assemblies (based on 132 structures shown in c and d). f) Number and intensity-weighted hydrodynamic diameters based on DLS of LT assemblies. Duplicate scattering spectra are shown for two independent samples.

The ability for LT assemblies to emulsify oil was examined using gasoline and Pennsylvania crude oil. The percentage of oil dispersion by emulsification is similar for both gasoline and crude oil, increasing with the total surfactant concentration (Figure 3.3a-c). The emulsions were O/W and exhibited creaming, indicating that the oil droplets were buoyant and rose to the oil/water interface at low surfactant concentrations or to the air/water interface at high surfactant concentrations. O/W emulsions are consistent with observations made by Riehm et al⁵³ for LT dispersant mixtures dissolved in ethanol and rich in Tween 80 (weight basis).

Figure 3.3: Oil emulsification in synthetic seawater with LT assemblies. a) Percent emulsified as a function of LT concentration; duplicate experiments are shown for gasoline (open squares) and crude oil (closed circles). b, c) Photographs of O/W emulsions taken 1 h after mixing with increasing surfactant concentration. d) Representative optical microscopy image of oil droplets stabilized by LT and different surfactant concentrations. The droplet size was similar across the surfactant concentrations for both gasoline and crude oil and ranged from approximately 10 to 30 μm .

Two regimes are clearly visible from the dispersion data in Figure 3.3a. In regime 1, the percent dispersion increases linearly up to approximately 0.12 mM surfactant, and in regime 2 the percent dispersion plateaus between 90-100%. Linear fits to these two regimes intersect at 0.12 mM for gasoline and crude oil (equivalent to 0.64 moles of surfactant per L of oil), which indicates that

chemical and physical differences between these two oils do not affect emulsion formation at the conditions examined. Images of oil droplets (gasoline) in water further show that the droplet density above the intersect (0.12 mM surfactant) was similar. It was also observed that the aqueous phases below the creamed emulsions became turbid as the LT concentration increased in regime 2. Increases in turbidity in the aqueous subphase were due to the presence LT assemblies when LT was in excess (relative to what was required for maximum dispersion).

The transition in percent dispersion at 0.12 mM from regime 1 to 2 suggests that this surfactant concentration corresponds to the minimum amount of surfactant needed to complete disperse the oils. At this condition, if the LT assemblies adsorbed onto the oil droplets and disassembled to form a surfactant monolayer, the monolayer would exhibit optimum geometric packing at the oil droplet interface. To test this geometric packing argument and confirm LT disassembly, we first calculated the minimum surfactant concentration, [surfactant], required to disperse oil with a known average droplet radius, r_d , from

$$[\text{surfactant}] = \frac{3V_{oil}}{r_d a_o N_{av} V_T} \quad (1)$$

where V_{oil} is the volume of oil, N_{av} is Avagadro's number, V_T is the total volume, and a_o is the average area per surfactant molecule. The average area per molecule was calculated as $a_o = x_L a_{oL} + x_T a_{oT}$, where x_L and x_T were the mole fractions of lecithin (0.6) and Tween 80 (0.4), respectively, and a_{oL} and a_{oT} were the areas per molecule for Tween 80 (0.86 nm^2)⁶⁴ and lecithin (0.78 nm^2)⁶⁵, respectively.

The minimum amount of surfactant needed to disperse the oil added to the system (equation 1) ranged from approximately 0.08 to 0.2 mM based on the droplet size range observed (10 to 30

μm). The experimentally determined concentration of 0.12 mM falls within this range and shows that the LT assemblies did in fact disassemble into well-mixed L+T monolayers. Rydhag and Wilson⁶⁶ have shown that emulsions stabilized by soybean lecithin require multiple phospholipid layers to remain stable. By adding Tween 80, which provided steric repulsion between dispersed oil droplets⁴, our oil emulsions were stable with a single LT monolayer.

When LT are co-dissolved in a solvent and added to an oil/water system, the combination of the low lecithin HLB and the steric repulsion between Tween 80 headgroups provides high oil dispersion and high emulsion stability⁴. Emulsion stability tests were conducted with aqueous L, T, and LT assemblies to determine if this synergistic feature is observed when the dispersant is present in the aqueous rather than being applied to the oil phase.

Lecithin was not effective alone as a dispersant consistent with rapid coalescence that been observed for lecithin-stabilized O/W emulsions (Figure 3.4a)⁵³. Phase separation was observed immediately and only a fraction of the oil remained dispersed in the aqueous phase, creaming at the oil-water interface. As shown by Nyankson et al⁴⁹, lecithin yields low emulsion stability due to its low HLB and oil solubility, which favors the formation of water-in-oil emulsions rather than O/W based on the Bancroft rule⁶⁷. Tween 80 was a more effective emulsifier, as shown previously when dissolved in a solvent and consistent with it favoring O/W emulsions, and the emulsion remained (creamed) after 1 day of preparation (Figure 3.4b). There was evidence of a foam layer over the duration of the experiment. The LT assemblies exhibited high initial oil dispersion with minimal foaming based on visual observation, and oil droplets were observed in the subphase after 30 min (Figure 3.4c). After the initial emulsion was formed, the emulsion creamed, as observed for Tween 80, and exhibited signs of phase separating after 1 day. The

long-term emulsion behavior (> 10 min) of LT and Tween 80 dispersions was similar, suggesting that steric repulsion provided by Tween 80 was primarily responsible for droplet stabilization.

Creaming rates for emulsions formed LT assemblies were estimated using Stokes' Law assuming an oil-water density difference of 0.2 g/ml. Based on the droplet size range observed, creaming rates from 4.4 to 39.6 ($\times 10^{-5}$) m/s correspond to complete creaming taking place within approximately 2-16 min. This is in agreement with emulsion creaming observed within 10-30 min of initial emulsion formation (Figure 3.4c).

Figure 3.4: Oil emulsification using a) lecithin, b) Tween 80, and c) lecithin-Tween 80 self-assemblies at 0.23 mM (0.022 wt%) total surfactant. d) Comparison of the fraction of oil dispersed from (a-c).

Finally, oil emulsion stability for LT assemblies is compared to Corexit 9500A. This comparison provides further insight into the ability for LT assemblies to disperse oil as previous work with LT dissolved in ethanol have been shown to provide greater emulsion stability relative to Corexit⁴. In this study the LT dispersant solution was prepared at 50 mM total surfactant and the

assemblies exhibited a similar size distribution as shown in Figure 3.2 when prepared at 2.5 mM. Hence, aqueous LT dispersants can be prepared over a range of concentrations while maintaining the assembly microstructure.

Crude oil emulsions prepared with LT assemblies were stable at 0.4 and 0.8 wt% surfactant (Figure 3.5, i and ii), and exhibited creaming consistent with emulsion behavior at lower LT concentrations (Figure 3.5a-c). Emulsions prepared with Corexit began to destabilize after 10 min, yielding separate oil phases at Corexit concentrations of 4 and 8% (Figure 3.5, iii and iv; concentrations based on surfactant analysis¹⁰). After 1 day there was no evidence of an emulsion phase and the volume of the oil phase increased with increasing dispersant volume. This increase in oil phase volume can be attributed to the organic solvents in Corexit mixing with the crude oil. The emulsion stability study shows that LT assemblies are more effective at stabilizing oil droplets and preventing coalescence than Corexit due to the strong adsorption of lecithin at the oil/water interface and the steric stabilization of droplets provided by the polar Tween 80 headgroup⁴.

Figure 3.5: Crude oil emulsion stability with LT assemblies (i, ii) and Corexit 9500A (iii, iv).

Conclusions

We have shown lecithin-Tween 80 mixtures form vesicles and disk-like micelles in water at room temperature, and that these structures can be used to emulsify gasoline and crude oil by adsorbing onto the oil droplets. The maximum amount of oil dispersion directly correlated to the amount of surfactant needed to completely cover the oil droplet interface. Results from this work show that LT assemblies can achieve similar levels of emulsion stability compared to ethanol-based LT dispersants, and that the O/W emulsions formed by LT mixtures are more stable than those formed with lecithin alone. In the context of treating oil spills, aqueous self-assemblies may be advantageous to surfactants traditionally solubilized in organic solvent packages because they eliminate the potentially toxic solvents. While aqueous-based dispersants would likely not be

effective in low mixing energy environments, they would be effective in high mixing energy environments such as rough sea conditions or deep-sea injection at well heads.

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CHAPTER 4 : MANUSCRIPT – II

Surfactant enhanced bioremediation of oil by *Alcanivorax borkumensis* using food grade amphiphiles

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Abstract

Oil spills pose a tremendous threat to the marine ecosystem and with that the livelihood and safety of humans that live near the sea. The Deepwater Horizon disaster of 2010 was a most recent example that caused disaster for thousands of people. After an oil spill occurs, however, we are not completely helpless—as there are methods that have been developed over the years to ensure that the oil can be removed and/or cause the least amount of environmental damage possible. One of the most prominent of these methods is the application of a chemical mixture known as a dispersant—a formulation of surfactants dissolved into a solvent, which is sprayed onto the oil slick and thus able to disperse the oil into small droplets in the water column due to the combined amphiphilic nature of the surfactants and the agitation of waves. The dispersion of the oil into the water column not only prevents the oil slick from floating to the coast, where marine life is more fragile and the ecosystem more populated, but has been shown to speed up the degradation process by increasing the interfacial area between the oil and water and thus the bioavailability for any oil degrading microbes that live within the water.

The most frequently used dispersant is Corexit 9500, 2.1 million gallons of which were applied to the oil spill in 2010¹¹. There are, however, some concerns regarding the environmental impact of Corexit 9500; studies showing the persistence of some of its components, such as DOSS, as well as the inhibition of bacterial growth. Due to these concerns, there have been calls for alternative dispersants that are more environmentally friendly yet just as effective. One such alternative dispersant that has been shown to exhibit similar dispersant effectiveness to Corexit 9500 has been developed using the phospholipid lecithin and the surfactant Tween 80.⁴

The aims of this study were to test the efficacy of a new, food grade dispersant mixture known as lecithin/Tween 80, and to see how it compares to the most common dispersant mixture

known as Corexit 9500 in promoting biodegradation by an oil degrading bacterium. Both of these dispersant mixtures were compared against lecithin alone, Tween alone and no dispersant at all in order to identify whether dispersant effectiveness, surfactant toxicity or surfactant biodegradability was what led to more degradation.

Our findings show that in each case where a dispersant was added, oil degradation was enhanced. After 144 days, the degradation percentages were as follows: 25% for a no dispersant control, 35% for a mixture supplemented with Corexit 9500, 38% for lecithin alone, 35% for Tween 80 alone, and 52% for lecithin/Tween 80 dispersant mixtures. In a direct comparison between the two effective dispersant mixtures, lecithin/Tween 80 and Corexit 9500, lecithin/Tween 80 proved to promote more degradation. Through microscopic analysis of these systems, it was found that each was capable of microbial adhesion and biofilm formation, and through these mechanisms and continual collisions, lead to microbe-oil agglomerates known as marine snow.

Introduction

Oil spills pose an enormous threat to coastal ecosystems and marine life⁵ as exemplified by the Deepwater Horizon disaster in 2010, where an explosion led to the discharge of approximately 200 million gallons of crude oil into the Gulf of Mexico.^{6,7} During the spill, a variety of methods were employed in order to combat the spill and mitigate the damage that the oil may cause to the marine environment. The method believed by many to be most effective at preventing coastal damage and promoting biodegradation is the application of chemical mixtures known as dispersants. A dispersant is a mixture of both amphiphilic compounds known as surfactants, which assemble and pack tightly at oil/water interfaces to lower the surface tension and disperse the oil into the aqueous phase, and organic solvents, which solubilize the surfactants and allows them to be sprayed onto a slick.⁹ When oil is dispersed in water as droplets the

specific surface area of the oil is increased, which increases the bioavailability to microorganisms capable of utilizing the oil as a food source (known as hydrocarbonoclastic bacteria).

Corexit, which is currently produced by Nalco Holding Company, is the most widely used class of these dispersant mixtures. During the Deepwater Horizon incident, two members of the Corexit family, Corexit 9500 and 9527, were applied to the oil at both the surface and at the wellhead at an amount upwards of 2.1 million gallons.¹¹ Corexit 9500 (CXT) is a mixture of the surfactants Span 80, Tween 80 and 85, and DOSS (dioctyl sulfosuccinate) dissolved in propylene glycol and petroleum distillates.¹⁰ Span and Tween 80 are both nonionic and have been shown to be biodegradable, while DOSS is anionic and has been shown to have adverse environmental impacts: irritation to the eyes and skin,¹² inhibition of crude oil oxidation by bacteria as well as bacterial growth,^{13,14} and persistence within the environment.¹¹

Due to the concerns surrounding CXT, there is interest to develop alternative dispersants; dispersants that are as effective as CXT in dispersing the oil and promoting degradation, but less toxic to marine life and human health. A proposed surfactant mixture using food grade amphiphiles has been shown to be just as effective as CXT in dispersing oil.^{4,19,68} This mixture, a combination of the phospholipid lecithin and the surfactant Tween 80, warrants further study to determine how effective it is at accelerating oil degradation. This is one of many necessary steps to determine whether lecithin/Tween 80 mixtures are commercially viable options to conventional dispersants.

In addition to high dispersion effectiveness, which increases bioavailability through emulsification, there are many factors of a dispersant system that impact bioremediation efforts. First is the potential toxicity and/or biodegradability of the chemicals (both surfactants and solvents) to the microorganisms that will be performing the degradation. Studies have shown that surfactants can inhibit biodegradation due to direct toxicity, and that the presence of a

biodegradable surfactant could help, but in some cases inhibit the biodegradation process.³⁴ In addition, some surfactants have the ability to change a microorganism's cell surface properties and hydrophobicity, and thus affect its ability to adhere to and uptake oil.³⁹ In turn, surfactant charge and structure can also change the oil/water interface when adsorbed and influence microbial attachment as well.³⁵ Finally, the species present within a microbial community that will be ultimately performing the biodegradation can lead to dispersant-specific results, as the ability to metabolize different chemicals is dependent on microbe species.⁶⁹

Alcanivorax borkumensis is a model hydrocarbonoclastic bacteria that have been used in prior studies to test the efficacy, biodegradability and toxicity of dispersant mixtures.^{14,27} The ubiquity of the *Alcanivorax* genus, and its prominence as the dominant group present after an oil spill event make it a prime candidate as a model oil degrading organism.^{20,70}

This study aims to determine how the addition of lecithin/Tween 80 (LT) mixtures promote oil biodegradation by *A. borkumensis*. LT dispersed mixtures are compared to controls, such as CXT and a mixture with no dispersant, and also to mixtures supplemented with lecithin or Tween 80 alone. By examining dispersants consisting of lecithin or Tween 80 alone, it is possible to relate the exceptional dispersion performance that has been reported due to the paired synergy to increased biodegradation (i.e. if enhanced biodegradation is attributed to greater oil bioavailability or reduced toxicity provided by the two food grade amphiphiles). A low oil concentration of 1 g/L was examined to more accurately correspond with the conditions of an oil spill due to the dilution that occurs at sea after dispersant application.⁷¹ Optical (bright and dark field) and fluorescence microscopy and UV-vis spectroscopy were used to examine the colloidal properties of the dispersion and the extent of bacterial attachment, biofilm formation and droplet aggregation. The extent of biodegradation was measured by GC-MS to connect the physical phenomena observed within the dispersions to bioremediation performance.

Materials and methods

Solution preparations

Dispersant formulation. The lecithin Tween 80 dispersant mixture used through the course of these experiments was composed of the two surfactants Tween 80 and lecithin (95% L- α -phosphatidylcholine, soy) (Avanti Lipids), dissolved within ethanol. The weight ratios were 20 % ethanol, 32 % tween and 48% lecithin. The lecithin/Tween-80 mass ratio chosen was used due to prior studies done that show it has the highest dispersion effectiveness.¹⁰ The surfactant/solvent ratio was used due to other studies which showed 80% surfactant mass ratio had the best efficiency in a baffled flask test.¹⁹ The lecithin alone and Tween 80 alone dispersants mixed were created at the same surfactant/solvent ratio as LT. Both were 80% surfactant by weight and dissolved in ethanol.

Sodium pyruvate. The sodium pyruvate medium used throughout the course of these experiments was taken from a recipe developed by Patricia Sobecky at the University of Alabama. The contents of the sodium pyruvate solution are as follows: 500 mL deionized water, 8.775 g NaCl, 0.375 g KCl, 3.015 g MgSO₄ anhydrous, 0.725 g CaCl₂ dihydrate, 2 g Tryptone, 1.5 g Yeast extract, and 5 g sodium pyruvate. These materials are mixed together on a stir plate until all the materials are dissolved and then sterilized by filtration through a 0.2-micron pore sized membrane (Corning Disposable Vacuum Filter/ Storage systems).

Nutrient supplemented artificial salt water (NASW). The nutrient supplemented artificial salt water (NASW) medium used throughout the course of these experiments was taken from a customized minimalized broth recipe used by the Tripathi group in the following papers.^{33,72} The contents of this solution are as follows: 500 mL deionized water, 9.725 g NaCl, 1.2 g CaCl₂ dihydrate, 0.275 g KCl, 1 g KNO₃, 0.063 g H₂KO₂P, 6.4 g MgCl₂ hexahydrate and 1.62 g MgSO₄ anhydrous.

Due to the fact that the salts interact with each other during dissolution (observed during preparation), this solution was split into four separate solutions where the salts were dissolved separately and then mixed together before being sterilized through filtration (same sterilization method as Sodium pyruvate media). The four mixtures are Solution 1 (200 mL water, CaCl_2 , NaCl and KCl), Solution 2 (100 mL water, KNO_3 , $\text{H}_2\text{KO}_2\text{P}$), Solution 3 (100 mL water, MgCl_2), and Solution 4 (100 mL water, MgSO_4).

Aseptic technique. Important to note is the fact that aseptic technique was followed while using these procedures. Any procedures involving sterile medium or bacteria were performed under a benchtop flame in order to prevent any contamination.

GC-MS oil. The oil used throughout this study was a mixture of hexadecane (a saturated hydrocarbon capable of metabolism by *A. borkumensis*) and phenanthrene (a polycyclic aromatic hydrocarbon unable to be metabolized by AB). The mass/mass ratio was 1/30, this being 1 mass unit of phenanthrene dissolved in 30 units of hexadecane.

Bacterial stock preparation

In order to ensure that the experiments being performed would be reproducible and also contain similar bacteria, it was necessary to grow up and store a bacterial stock in the freezer which would be collected over time to perform the experiments.

A. borkumensis was first streaked on a plate and sent to URI by collaborators at Tulane University. This plate was placed in the refrigerator for storage. To grow up the bacterial stock, bacteria were scraped off the plate using an inoculating loop and placed into an autoclaved Erlenmeyer flask containing 50 mL of sterile sodium pyruvate solution. This culture was then placed into an incubator at 25 °C and rotated on a shaker table at 120 RPM. This was left alone until the absorbance reading of the culture reached 1.2 (at approximately 72 hours).

Once it had reached an absorbance reading of 1.2, 9 mL of a 1:1 volume ratio DMSO:Glycerol mixture was added to the culture and the culture was swirled by hand for 20 seconds. This mixture was then transferred 500 microliters at a time to mini centrifuge tubes, and these mini centrifuge tubes would be stored in the freezer and kept as bacterial stock.

Baffled flask oil dispersion (without bacteria)

Dispersion effectiveness was examined in baffled flasks without bacteria present (see figure 4.1). A thin layer of oil equivalent to 1 g/L oil in the system was placed on 50 mL of NASW in sterilized 125 mL baffled Erlenmeyer flasks. Dispersant was added at a 1:10 dispersant to oil ratio and the flasks were placed on a shaker table operating at 120 RPM in an air incubator at 25 °C. Turbidity, reflecting oil droplet formation and colloidal stability, and oil droplet morphology were examined over time by UV-vis spectroscopy and microscopy, respectively.

Figure 4.1: Schematic depicting dispersion formation in baffled flask experiments.

Baffled flask oil dispersion and biodegradation (with bacteria)

The bacterial suspension to be placed in the experimental flasks was first grown in sodium pyruvate media by inoculating 50 mL of Sodium Pyruvate solution with one frozen bacteria stock (Figure 4.2). This inoculation was performed in a 125 mL sterilized baffled Erlenmeyer flask, and after inoculation the flask was placed on a shaker table at 25 °C rotating at 120 RPM. These bacteria were then grown to an absorbance value of 0.45 (exponential phase),

which took approximately 40 hours on average. The solution was transferred to a 50 mL centrifuge tube and centrifuged at 5000 RPM (2460 RCF) in an Eppendorf 5430 centrifuge for 10 minutes. The supernatant was removed and replaced with 50 mL of clean NASW. The tube was then shaken vigorously to resuspend the bacteria.

Figure 4.2: Schematic of bacterial preparation for dispersion and biodegradation experiments (not to scale).

The bacteria suspension was then centrifuged for a second time and the supernatant recovered. At this step, instead of adding 50 mL of NASW, the NASW volume was adjusted to yield an absorbance of 0.5. This was typically achieved with 30 mL of NASW. Dispersion and biodegradation experiments were then conducted as shown in Figure 4.1. The aqueous phase consisted of 40 mL of NASW and 10 mL of the bacterial suspension to yield an initial absorbance of 0.1. To the flasks, 65 μ L of hexadecane (yielding 1 g/L) and then 6.5 μ L of dispersant were added to the system. The flasks were placed on a shaker table operating at 120 RPM in an air incubator at 25 °C. Photographs of these flasks during an experiment can be seen the Appendix in Figure A.3.

Characterization of dispersed oil-bacteria mixtures

Absorbance studies. All absorbance readings were taken with a Cary 50 UV visible spectrophotometer. Readings were taken by first recording a zero with clean media and then collecting the read of the sample. All readings were performed at a wavelength of 600 nm, in polystyrene cuvettes and using 2 mL of sample.

Bright field microscopy. For bright field microscopy, 10 μ L of sample was taken from the bulk phase of an experimental flask and then placed on a microscope slide. No cover slip was used for these samples in order to prevent any droplet adhesion to the cover slip that may interfere with properties of the mixture being observed. Bright field microscopy was performed with a Fisher scientific Micromaster Microscope.

Fluorescence microscopy. For fluorescence microscopy, 100 μ L of sample were taken from the experimental flask bulk phase and then mixed with 25 μ L of a BacLight dye solution prepared beforehand (6 μ L of Propidium Iodide and Syto 9 found in BacLight kit, mixed with 1 mL of DI water). This mixture was then incubated in the dark for 15 minutes. After the incubation, the sample was analyzed with the CytoViva dark field microscope.

Dark field microscopy. For dark field microscopy, 10 μ L of sample was taken from the experimental flask bulk phase and placed on a microscopic slide. A cover slip was then placed carefully on top. Analysis was also done with the CytoViva dark field microscope.

Oil degradation quantification by GC-MS

Extraction and sample preparation. At a given time point for degradation analysis, an entire flask is sacrificed. At that point, the 50 mL of mixture within the Erlenmeyer flask is transferred into two 50 mL polypropylene centrifuge tubes, at 25 mL each. Then 25 mL of Dichloromethane (DCM) is transferred into each centrifuge tube (after using each 25 mL of DCM to rinse the Erlenmeyer flask for any residual oil). Each tube is mixed by hand rigorously for 30

seconds before placing them into an Eppendorf Centrifuge 5430 at 7500 RPM (5534 RCF) for 10 minutes. Once the centrifugation is finished, the aqueous layer and biofilm is discarded, and the remaining DCM extract is passed through anhydrous Magnesium Sulfate in order to remove any water. This final extract is readjusted to 50 mL, and then diluted with a dilution factor of 10. These methods were modified from a study performed by Mohanty et al.³⁹

GC-MS. Samples diluted with DCM were placed in an autosampler and 1 μ L was injected into the column running in splitless mode. The carrier gas used was helium, the column oven temp was 50 °C and injection temperature was 200 °C. The ion source temperature was 175 °C and the interface temperature was 200 °C.

Once the sample was injected, the column was held for 1 minute at 50 °C. Then the oven temperature was raised at a rate of 10 °C/minute to 100 °C, where it was held for 1 minute. The oven temperature was then raised at a rate of 8 °C/minute to 200 °C held again for 1 minute.

Quantitative analysis of GC-MS. After the data was collected from the GC/MS, data was analyzed in the MestReNova program. In this program, the peak of hexadecane was identified and then the peak area was found. These peak areas were converted to concentrations using previously obtained calibration curves.

Results and discussion

Results are first presented for dispersion effectiveness to depict the oil dispersion properties in the absence of bacteria. These results are compared to the dispersion properties with bacteria to examine the impact of bacteria adhesion onto droplets, biofilm formation, and droplet agglomeration. With this approach it is possible to determine how bacteria modify the state of oil droplet dispersion and connect this to biodegradation performance.

Dispersion effectiveness

As seen in Figure 4.3a, LT and CXT oil dispersions exhibited high absorbances consistent with high dispersion effectiveness. For CXT, there was a large rise in absorbance from 0 hours, when the dispersant was first added, to 1 hour indicating rapid oil dispersion relative to the other dispersants examined. CXT provides extremely low oil/water interfacial tension leading to near-immediate oil dispersion with low mixing energy. LT requires more mixing time to achieve high oil dispersion based on the initial absorbance rise. At 10 hours both LT and CXT form stable dispersions that persisted over 110 hours based on the absorbances. The droplet sizes at 48 hours were approximately 1 to 20 μm for LT and CXT. A slight reduction in absorbance was observed after 72 hours possibly due to droplet coalescence.

Figure 4.3: Absorbance readings and bright field microscopy of dispersed oil. a) Absorbance over time. Dispersant was added to the oil film at time = 0 hours. Microscopy images shown for oil droplets dispersed by b) LT, c) CXT, and d) lecithin at 48 hours. Error

bars for the LT and CXT mixtures show error of duplicate experiments. Scale bar is equal to 100 μm .

Tween 80 alone was not an effective dispersant. The dispersant effectiveness is estimated near 0% based on the absorbance, which remained near 0 over 110 hours (Figure 4.3a), and microscopy analysis revealing very few droplets (not shown). The negligible dispersant effectiveness at these conditions can be attributed to the high hydrophilicity or water solubility of Tween 80,⁴ which is ineffective at creating stable emulsions of oil in water at these mixing conditions. In prior experiments, it has been shown that Tween 80 increases the bioavailability of oil to microorganisms through micellar solubilization rather than oil droplet formation or emulsification.³⁰ A description of each process by which bacteria can access oil in surfactant supplemented systems can be seen in Figure 2.13 of the Background section of this thesis.

Lecithin alone was a more effective dispersant than Tween 80, but far less effective than LT. The absorbance for oil dispersions using lecithin increased modestly over time and microscopy analysis revealed that a small number of large droplets were formed (as opposed to many small droplets formed by LT and CXT). Upon further investigation using bright field microscopy, many of these droplets were shown to have non-spherical shapes pointing to droplet coalescence. Lecithin is very hydrophobic and because of its oil solubility it prefers to form water in oil emulsions as opposed to oil in water emulsions. Hence, the dispersion is not stable and droplet coalescence leads to large oil droplets that cream and rise to the water surface.⁶⁷

These results show, in concordance with other studies, that the combination of lecithin/Tween 80 is much more effective at dispersing oil than each surfactant (lecithin or Tween 80) alone. Athas et al⁴ showed that crude oil emulsions dispersed by lecithin or Tween 80

demulsified within 30 minutes after vortex mixing. In this same study, the lecithin/Tween 80 dispersions performed exceedingly well and did not demulsify after 30 minutes.

The synergy between lecithin and Tween 80 is explained in two main ways.

- 1) Through the Hydrophilic Lipophilic balance (HLB) of both molecules, where surfactants are valued according to the ratio between their hydrophilic and lipophilic components. In the case of emulsifying petroleum/ crude oil it has been shown that the best combined HLB number of the surfactants is between 9 and 11. Lecithin has an HLB of 8, and is oil soluble while Tween 80 has an HLB of 15 and is very soluble in water. A combination of these components at a 60/40 mass ratio puts the HLB at about 10.8, an effective number for emulsification.
- 2) Synergistic combination of the lipophilicity of lecithin that provides tight packing at the oil/water interface and the hydrophilic bulky head group of Tween 80 that provides steric repulsion between droplets to prevent coalescence, which can lead to creaming (large droplets with low density driven to the water surface via buoyancy).

Dispersion effectiveness and bioremediation

There are four ways considered in which bacteria can impact the absorbance measurements: the bacteria themselves scatter light and contribute to absorbance, the bacteria inhibit or promote oil droplet formation during dispersion, the bacteria cause droplet agglomeration as they proliferate at the oil droplet interface and form biofilms, and the bacteria can degrade the oil.

The UV-vis absorbance behavior for the systems with bacteria differed from those without bacteria, as can be seen by comparing Figure 4.3a (without bacteria) and Figure 4.4 (with bacteria). One characteristic that was similar was the rapid initial rise of CXT compared to LT. At 1 hour for the mixtures with bacteria present the CXT dispersion reached an absorbance of 0.9,

much higher than that for LT (absorbance of 0.3). This shows that the bacteria, despite their ability to adhere to and stabilize oil droplets, do not inhibit oil dispersion by CXT and LT. Another important note is that the absorbance at the time point zero of these graphs is 0.1, opposed to an absorbance near 0 in the graphs without bacteria. This value of 0.1 is attributed to the presence of bacteria in the aqueous phase, which was confirmed by the control experiments (no dispersant added). Based on the control, it is apparent that light scattering is dominated by oil droplets and/or oil droplet-bacteria agglomerates in when dispersants are used as opposed to the bacteria themselves.

Figure 4.4: Absorbance at 600 nm of dispersant mixtures with bacteria over time. For the control condition, oil was added but no dispersant. Error bars show the error of duplicate experiments.

One notable observation in Figure 4.4 is that the absorbance of the LT dispersion decreases exponentially after 12 hours, while the CXT dispersion absorbance remains nearly constant. Figure 4.5 gives further insight into how the LT and CXT dispersed mixtures are behaving from Day 2 to Day 4 and shows how the systems are evolving over time. Clearly, one can see that the LT dispersed mixtures are losing droplets to either degradation or aggregation while the CXT droplets persist throughout the 4 days.

Figure 4.5: Bright field microscopy of LT and CXT mixtures over time. Scale bar equal to 100 μm .

Figure 4.4 also shows the absorbance over time graphs for lecithin alone, Tween 80 alone and the no dispersant control. For Tween 80, there is also a rise but it is slower and over a longer period of time. Microscopic analysis showed no droplets dispersed in the oil phase. The absence of droplets suggests that not only was the Tween 80 surfactant incapable of forming an emulsion, but that the Tween 80 also prevented the bacteria from breaking off any droplets, which occurs in the no dispersant system (and can be seen in Figure 4.6). This observation agrees with some prior studies, which have shown that Tween can impede bacteria attachment to an oil/water interface.³⁷ Therefore, the rise in absorbance seen in the Tween 80 system could be attributed to bacterial growth through either utilization of dissolved Tween 80 as a food source, which has been shown to promote bacterial growth of *A. borkumensis*,²⁷ or due to the uptake of oil with micellar solubilization, which has also been reported for Tween 80.³⁰

Lecithin alone mixtures have an absorbance rise in the beginning, indicating at least partial dispersion of oil into the aqueous phase. The partial dispersion was confirmed by the presence of droplets seen in Figure 4.6. When examining these mixtures under the microscope, it was possible to see a number of interesting structures, matching up with the UV-vis results that indicate some dispersion of the oil had occurred. The structures found in these images had a rough shape to them, and looked much different than the droplets formed in the case of LT or CXT. The droplets were also larger than either LT or CXT dispersed oil droplets.

The control samples have two major differences in comparison to the samples with dispersant present. The first difference is that the number of droplets present in solution is exceedingly lower (albeit more than Tween 80). However, there are still some droplets that are able to form. The formation of these droplets suggest bacteria's production of EPS and biosurfactant and/or the adsorption of bacteria to the oil/water interface and thus creation of droplets.⁷³

Figure 4.6: Bright field microscopy of no dispersant (control) and lecithin mixtures at 48 hours. Scale bar for no dispersant is 50 μm and scale bar for lecithin is 100 μm .

Agglomerates seen with Dark Field and Bright Field Microscopy

Through dark field and fluorescence microscopy, it was possible to obtain detailed images of the oil droplet-bacteria agglomerates (Figure 4.7, 4.8 and 4.9). In the cases of LT, lecithin, CXT and no dispersant mixtures, agglomerates were observed, while for Tween 80 neither droplets or agglomerates were observed due to Tween 80's inability to perform dispersions at these conditions and also possibly preventing bacteria adhesion at the oil/water interface. Microbial oil agglomerates are known to be formed with sweeping flocculation and the bridging effect.⁴⁶ When these agglomerates become quite large, on the order of 10^2 to 10^4 μm , they are known as marine snow. Marine snow was found in large quantities after the BP oil spill.

Figure 4.7: Fluorescence microscopy over time. Scale bar equal to 20 μ m

Figure 4.7 shows a side by side of the structures formed in no dispersant, LT, and CXT. As one can see, the LT and CXT mixtures are more adept at forming microbe-oil agglomerates than the mixtures without dispersant, but there is still bacteria adhesion that occurs in the no dispersant (control) mixture. As time goes on, the agglomerates get larger or grow presumably due to the increased EPS concentration, bacteria growth or biofilm formation, and more opportunity for droplet/bacteria collision. Fluorescent microscopy of lecithin alone mixtures showed some droplets colonized by bacteria, while Tween 80 alone showed no droplets with a large amount of healthy, free floating bacteria. Figure 4.8 shows an image of both these mixtures at 48 hours.

Figure 4.8: Fluorescence microscopy of lecithin alone and Tween 80 alone at 48 hours. Scale bar equal to 20 μm

Figure 4.9 also shows agglomerates, this time of the larger variety collected at the 9 and 10-day mark. Agglomerates were observed through bright field, fluorescent and dark field microscopy. The bottommost image for both LT and CXT also indicates that the dye had no major effect on the formation of agglomerates, ruling out the possibility that the dye was influencing bacteria and colloidal behavior.

Figure 4.9: Microscopy of agglomerates for LT and CXT at Day 9/10. Scale bar for bright field images is equal to 100 microns. Scale bar for dark field/fluorescence is equal to 20 μm

Figure 4.10 shows how agglomerates of both LT and CXT mixtures appeared at 9 days. Larger droplets were observed in the LT agglomerates compared to the CXT agglomerates, and a large majority of the observable droplets formed by LT were associated with agglomerates while

droplets formed by CXT were observed both within and outside of agglomerates (Figures 4.10b and d).

Figure 4.10: Bright field microscopy at 9 days. Scale bar equal to 100 μ m. a) LT agglomerate, b) Space between agglomerates in LT mixture, c) CXT agglomerate, d) space between agglomerates in CXT mixture

Biodegradation

GC/MS results, seen in Figure 4.11, display the oil biodegradation percentages for each dispersant mixture. As can be seen, all dispersant mixtures enhance biodegradation in comparison to the no dispersant control for all time intervals. The no dispersant control reaches 25% degradation after 144 hours. Comparatively, LT yields 52% degradation, CXT 35% degradation,

lecithin 38% degradation, and Tween 80 35% degradation. The fact that all mixtures, regardless of dispersant choice, “flatline” at about 48 hours, suggests that there could be another limiting nutrient in the system that ran out at this time point (possibly nitrate or phosphate).

Figure 4.11: Biodegradation percentage over time for each dispersant mixture

The results shown here agree with the UV-vis spectroscopy and microscopy data shown earlier. The absorbance graph shown in Figure 4.4 and the lack of free oil droplets for the LT system (in comparison to CXT) in Figures 4.5 and 4.9 indicated that the LT system had a large amount of degradation, which is confirmed through the GC/MS data in Figure 4.11.

Conclusions

The goal of this study was to elucidate the effects that dispersant formulation had on bacterial behavior, time evolution of the dispersions, and the ultimate degradation percentage. Specifically, we were interested in the newly formulated lecithin/Tween 80 mixture, and we wished to compare it with Corexit 9500, the most widely used dispersant in the field. Prior experiments that

had been performed suggested that dispersant specific properties such as dispersant effectiveness, surfactant utilization/toxicity, oil uptake/mechanism and surfactant charge all could play a role in the efficacy of a surfactant enhanced bioremediation effort. Other studies suggested that certain components of CXT could inhibit bacterial growth.

Through the results of this study we have found that LT was able to exhibit a dispersant effectiveness comparable to CXT. This was while lecithin alone exhibited a meager dispersant effectiveness and Tween 80 none at all.

We have also shown that the systems studied are very favorable for bacterial attachment and agglomerate formation. In the systems of lecithin alone, LT, CXT and even no dispersant, agglomerates were found. This finding agrees with the well-studied product of oil spill dispersion known as marine snow. In the cases of CXT and LT, where many droplets were present in solution, there were agglomerates found with multiple droplets attached.

In a direct comparison between the two best performing dispersant mixtures: LT and CXT, we have shown that CXT droplets persist longer. This can be explained by either the higher capacity of LT dispersed mixtures to agglomerate (a higher stickiness of these droplets) or a better degradation ability in LT systems. This better degradation ability could be explained by CXT's inhibition of bacterial growth seen in other studies.¹⁴

GC/MS analysis shows that the addition of a dispersant mixture enhances the degradation percentage substantially. This was the case for all four dispersant mixtures tested. Even Tween 80, which does not emulsify the oil (as shown by the lack of oil droplets in microscopy images) but has been shown to use micellar solubilization to transport it in prior studies, showed an enhancement in biodegradation. The degradation percentages at 144 hours were as follows: 25 % for the control; 35 % for CXT, 52% for LT, 38% for lecithin alone, and 35% for Tween 80 alone.

The following images conclude/ summarize what has been learned about each dispersant system and how each system evolves over time:

Figure 4.12: Summary of no dispersant mixtures

Figure 4.13: Summary of lecithin alone mixtures

Figure 4.14: Summary of Tween alone mixtures

Figure 4.15: Summary of lecithin/Tween 80 mixtures

Figure 4.16: Summary of Corexit 9500 mixtures

It is clear that surfactant choice plays a major role in not only the ultimate rate of degradation but also the mechanism of degradation and how the overall mixture behaves. While this study and others like it have shed some light on the complex relationship that exists, there is still much to be learned. Studies looking at different bacteria populations, oils, and surfactant concentration could all give more insight into these complex systems.

Ultimately, the recently developed LT mixture continues to show its potential in having better efficacy compared to CXT in promoting dispersion and microbe-oil agglomerate formation and having slightly better oil degradation in a 144-hour period. It is a promising formulation that deserves further study and could be an effective substitute to CXT while eliminating some of CXT's more controversial components such as DOSS.

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APPENDICES

1. Absorbance modeling

1.1 Absorbance as a function of droplet radius

Goal: In this model, we wished to understand the relationship between droplet size and absorbance assuming the same dispersed oil ratio.

Why? In order to pull even qualitative information from the UV-vis spectroscopy results, it was necessary to understand how agglomerate formation, degradation and droplet size would affect the absorbance reading.

Parameters:

Parameters	Value
Path length (microns)	10000
Refractive index (hexadecane)	1.434
Refractive index (water phase, NaCl conc of 0.02)	1.337
Dispersed oil ratio	0.0013
Wavelength of light (nm)	600

Using the above parameters and the equations found in the background section of this thesis (for Turbidity, Transmittance and Absorbance), the following table was able to be generated:

Drop radius (micron)	$Q_s=f(r, \lambda, n)$ (Mieplot)	Turbidity (calculation)	Transmittance	Absorbance
0.5	0.4999	0.000974805	5.84084E-05	4.233524324
1	1.7306	0.001687335	4.69891E-08	7.328002796
1.5	2.9107	0.001891955	6.07217E-09	8.216656165
2	3.3796	0.001647555	6.99455E-08	7.155240451
4	1.6908	0.000412133	0.016223005	1.789868706
5	2.4596	0.000479622	0.008260914	2.08297188
6	2.2062	0.000358508	0.027734587	1.55697829
7	1.8174	0.000253138	0.07954928	1.099363745
8	2.2257	0.000271257	0.066365902	1.178054997
10	1.8724	0.000182559	0.161122554	0.792843663

20	2.0555	0.000100206	0.367123766	0.4351875
50	1.986	0.000038727	0.678907765	0.168189224

Qs was found through the use of MiePlot version 4613.

Figure A.1: Mathematical relationship between droplet radius and absorbance

Conclusion: Complex relationship between droplet size and absorbance. With absorbance highest close to 1 micron and then becoming smaller when the droplets are either larger or smaller than this size.

1.2 Absorbance over time with a constant oil degradation rate

Goal: To find the relationship between time and Absorbance as a monodisperse oil dispersion is being degraded.

Why? Since the absorbance actually increases as the droplet size gets smaller, I was interested to see if the absorbance could increase while degradation is happening (and the droplets are shrinking). Of course, as the droplets are degraded, the dispersed oil ratio is also being affected

(but I wanted to see for sure how the relationship of time and absorbance would look over a given degradation process).

Parameters:

Parameters	Value
Path length (micron)	10000
Refractive index (hexadecane)	1.434
Refractive index (water phase, NaCl conc of 0.02)	1.337
Dispersed oil ratio at t=0	0.0013
Wavelength of light (nm)	600
Volume of oil eaten per time (microliter/day)--<assumption	6.5
Radius of droplet (micron)	10
Volume of one oil drop (micron ³)	4188.7867
Total volume oil (micron ³)	6.5E+10
Number of droplets	15517620
Volume of oil eaten per drop per time (microliter/day)	4.189E-07
Micron cubed/per drop	-418.8787

Variables: r is radius, V is volume, C is a constant, N_{drop} is number of droplets, Φ is dispersed oil ratio, V_{water} is volume of water

Constant degradation: $\frac{dV}{dt} = C \rightarrow dt = \frac{dV}{C}$

Degradation of one individual drop: $dV_{drop} = \frac{dV}{\# \text{ of drops}}$

Change in radius of sphere as volume changes: $\frac{dV}{dr} = 4\pi r^2 \rightarrow dr = \frac{dV}{4\pi r^2}$

Change in radius as time changes: $\frac{dr}{dt} = \frac{\frac{dV}{4\pi r^2}}{\frac{dV}{C}} = \frac{C}{4\pi r^2}$

$$\int_{r_0}^r r^2 dr = \frac{C}{4\pi} \int_0^t dt$$

If this integral is done out and the equation rearranged to solve for r, the solution is Equation 6.1.

$$r = \sqrt[3]{\frac{3Ct}{4\pi} + r_o^3} \text{ Equation 6.1}$$

$$\phi = \frac{\frac{4}{3}\pi r^3 N_{drop}}{V_{water}} \text{ Equation 6.2}$$

Using the parameters listed above and Equation 6.1, 6.2 and the equations for Turbidity, Transmittance and Absorbance listed in the background section of this thesis, it was possible to create the following table. Qs was generated using MiePlot version 4613.

Time (day)	Drop radius (micron)	Qs	Dispersed oil fraction	Turbidity	Transmittance	Abs
0	10	1.8709	0.0013	0.000182413	0.161358368	0.79220
1	9.654893846	1.9329	0.00117	0.000175675	0.172605585	0.76294
2	9.283177667	2.0712	0.00104	0.000174028	0.175470665	0.75579
3	8.879040017	2.2527	0.00091	0.000173157	0.177006328	0.75201
5	7.93700526	2.2006	0.00065	0.000135163	0.258817046	0.58700
7	6.694329501	1.8277	0.00039	7.9859E-05	0.449963106	0.34682
9	4.641588834	2.2037	0.00013	4.62903E-05	0.629453401	0.20103
9.5	3.684031499	1.6274	6.5E-05	2.1535E-05	0.806258912	0.09352
9.75	2.924017738	2.3673	3.25E-05	1.97341E-05	0.820910433	0.08570
10	6.10352E-05	0	2.95586E-19	0	1	0

Figure A.2: Function of absorbance with a constant degradation rate

Conclusion: The resulting model shows that as the oil is degraded, the absorbance will drop (not taking scattering due to bacteria into account).

2. Photograph of Baffled Flask set up (at conditions of Manuscript 2)

Figure A.3: Pictures of flasks at 24 hours