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E15PG00037: Multifunctional Herding-Sorbent Agents for Use in Icy Water

Final Report

May 2017

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U.S. DEPARTMENT OF ENERGY

Prepared for the U.S. Department of Energy
under Contract DE-AC05-76RL01830

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Preface

This study reported herein was funded by the U.S. Department of the Interior, Bureau of Safety and Environmental Enforcement (BSEE) through an Interagency Agreement, BSEE Contract E15PG00037, "Multifunctional Herding-Sorbent Agents for Use in Icy Water," with the U.S. Department of Energy, Pacific Northwest National Laboratory. This report describes Pacific Northwest National Laboratory researchers' development of a product—an "aggregator"—for use in oil spill mitigation to sorb spilled oil on seawater surfaces and facilitate in situ clean-up burns.

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Summary

This report details the development and testing of next-generation materials for oil spill mitigation—herein referred to as “aggregators”—for Bureau of Safety and Environmental Enforcement project E15PG00037. The development of the aggregator product, effects on performance by addition of biodegrading organisms, *in-situ* biodegradation rates, *in situ* burn performance at the Joint Maritime Test Facility, and herding and sorbent performance in icy water are highlighted below.

- *Aggregator development*. Significant gains were made in the development and characterization of aggregators using thermal treatment and fatty-acid modification of 40-mesh pine sawdust (wood flour) for use as an aggregator. Initial development and preliminary characterization of pine, poplar, fir, maple, and rice hull as a aggregator starting materials were assessed. Additionally, wood particle size was evaluated as a factor in aggregator sorbent performance. Thermal treatment was eventually removed for consideration as an aggregator manufacture methodology because it was not sufficiently buoyant in seawater. Fatty-acid modification of wood flour was selected as the best method for producing the aggregator, because the resulting product displayed buoyancy in seawater for months, absorbed oil, and repelled seawater. 40-mesh pine wood flour was selected because it had the best oil sorption of all wood species tested, and is commercially available in bulk quantities at low cost.

- *Biodegrader additives*. The viability of combining oil-degrading microorganisms with aggregator was assessed. Three representative oil-degrading microorganisms—*Alcanivorax borkumensis, Pseudomonas putida* and *Hormoconis resinae*—were evaluated for their viability at three different storage temperatures and for four different storage periods. Of all temperatures tested 17ºC was the most efficient temperature for preserving microorganisms. The filamentous fungus *H. resinae* was the most viable of all tested microorganisms; therefore, it is the recommended microorganism for combined long-term storage with the aggregator. A series of experiments was performed to determine whether infusing microorganisms with the aggregator altered its performance by assessing oil sorption capacity, hydrophobicity, and buoyancy. The addition of microorganisms to the aggregator resulted in slightly reduced oil sorption by approximately 5% at optimum conditions. Aggregator buoyancy and hydrophobicity of the aggregator were not affected.

- *Biodegradation*. Experiments were conducted to evaluate oil biodegradation in the presence of sawdust, 40-mesh oleic acid-modified pine aggregator, or no material added, and whether the addition of trace amounts of crude oil would prime oil-degrading microorganisms to accelerate oil biodegradation. Hydrocarbon analysis showed that the addition of the aggregator facilitated oil biodegradation, but the extent of any benefit is difficult to ascertain from this limited study. A 1-hour priming of microorganisms with crude oil did not show enhanced oil biodegradation; further optimization of priming methodology may be required to determine if this is a feasible strategy.

- *Burn tests*. A series of burn tests were conducted at the Joint Maritime Test Facility JMTF to assess the performance of the aggregator in large-scale burns. The tests used oleic acid-modified pine wood flour aggregators (mesh size 40); and two different crude oils, Dorado and Alaskan North Slope. A range of crude oil:aggregator ratios were evaluated to determine the optimal ratio for sorbing oil and supporting/enhancing combustion. An oil:aggregator ratio of ~1:10 exhibited the highest combustion efficiency. Additionally, a crude oil slick of 1 mm thickness could be ignited by adding aggregator to the slick. The aggregator acted as a wick pulling oil from the seawater surface, which allowed for combustion of thinner oil slick thicknesses. Although the aggregator showed great potential for igniting crude oil slicks at or below 1 mm of thickness and increasing the burn temperature, it displayed limited oil herding capability. Localized contraction of the oil was observed when the aggregator was dispensed to the surface, but it did not affect the entire oil slick as would a traditional liquid herding agent.

- *Herding and sorption in icy water*. The aggregator was tested for oil sorption in the presence of frazil sea ice. Oil sorption by the aggregator was not impeded by the presence of ice; furthermore, ice nucleation was not observed on the aggregator over a 48-hour test period. During burn tests, ice did not show a negative impact on the ability to burn oil in the presence of aggregator. Additionally, burn tests with the aggregator showed an increase in maximum flame height and burn rate compared to oil-only tests.

In summary, we have successfully developed an aggregator that sorbs oil on a seawater surface and facilitates *in situ* burns. The material is oleophilic, hydrophobic, buoyant in seawater for many months, and is unaffected by turbulence (e.g., by wave or wind action) that otherwise reduces the effectiveness of oil herders currently in use. The product has shown particular promise in facilitating *in situ* burns that are 1 mm or less in thickness. Furthermore, the product is non-toxic and can be infused with microorganisms to support bioremediation. This project started at a Bureau of Safety and Environmental Enforcement Oil Spill Response Technology Readiness Level (TRL) of 1 with a basic principle to be investigated and progressed to a TRL of 3 with the proof of concept demonstrated and data generated at completion.

Acronyms and Abbreviations

°C	degrees Celsius
°F	degrees Fahrenheit
ANS	Alaskan North Slope
ASTM	American Society for Testing and Materials
BSEE	Bureau of Safety and Environmental Enforcement
CA	contact angle
cDNA	complementary deoxyribonucleic acid
cm^{-1}	centimeter(s) per year
DTG	differential thermogravimetric (analysis)
ft	foot(feet)
FTIR	Fourier transform infrared (spectroscopy)
g	gram(s)
Hz	hertz
hr	hour(s)
ISB	in situ burning
JMTF	Joint Maritime Test Facility
kV	kilovolt(s)
µl	microliter(s)
L	liter(s)
µm	micrometer(s)
m	meter(s)
MA	marine agar
mg	milligram(s)
min	minute(s)
mL	milliliter(s)
mm	millimeter(s)
MOC	medium only control
MSL	Marine Sciences Laboratory
nm	nanometer(s)
NTC	no-template control
OA	oleic acid
OD	optical density
ORCAA	Olympic Region Clean Air Agency
PAH	polycyclic aromatic hydrocarbon
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PDA	potato dextrose agar
PNNL	Pacific Northwest National Laboratory
qPCR	quantitative polymerase chain reaction
RH	relative humidity
RNA	ribonucleic acid
RPD	Relative Percentage Difference
RPM	rotations per minute
RT-PCR	reverse transcriptase polymerase chain reaction
TGA	thermogravimetric analysis
TRCO	Texas raw crude oil (West Texas Intermediate)
TRL	Technology Readiness Level
WPG	weight percentage gain
WTI	West Texas Intermediate
XRD	X-ray diffraction

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1.0 Introduction

Herding agents are low-viscosity chemicals that change the interfacial forces of the oil-water interface, causing separate patches of oil to be attracted to each other and form thicker slicks. Herding agents are used to push oil slicks in a direction or to concentrate oil into a smaller surface area with a slick thickness suitable for in situ burning (ISB) or skimming (SL Ross 2010; Buist et al 2011; SL Ross 2012). However, typical herding agents only have a single function, limited efficacy, and work within a limited range of environmental conditions. Also, many herding agents are composed of synthetic chemicals that might not be ideal for ecologically friendly oil cleanup.

Sorbents are used to concentrate oil by absorbing the oil into their interiors and/or adsorbing oil onto their surfaces. Once saturated, sorbent materials can be collected and the oil recovered, disposed of, or burned. Wood flour and other organic materials (e.g., feathers, ground corncob, hay, peat) have been previously used as sorbents, but they typically absorb water as readily as oil and as a result, many are prone to sinking (Adebajo et al. 2003; NRT-RRT Factsheet 2007). Natural inorganic sorbents (e.g., clay, perlite, vermiculite) sink even faster (Asadpour et al. 2013). Synthetic sorbents are also available, but many of these contain plastics and polymers that are undesirable in the environment, or novel materials that are still expensive to produce (e.g., aerogels); other problems are related to the inability to separate oil from the sorbent after collection (Adebajo et al 2003; NRT-RRT Factsheet 2007; Olalekan et al. 2014, Xu et al. 2015).

A number of studies have investigated the ability to modify natural sorbents, particularly materials sourced from wood, straw, or cane, to render these more hydrophobic (water repelling) and oleophilic (oil attracting) for oil capture (see for example Ael-A et al. 2009; She et al. 2010; Karan et al.2011; Teli and Balia 2013a; Teli and Balia 2013b; Zhang et al. 2014). The objective of the Pacific Northwest National Laboratory (PNNL) project reported here was to develop and synthesize a new agent by chemically modifying inexpensive and readily available materials so that it could have both herding and sorbent functions. This study was funded by the U.S. Department of the Interior, Bureau of Safety and Environmental Enforcement through Interagency Agreement E15PG00037 with the U.S. Department of Energy, Pacific Northwest National Laboratory.

1.1 Project Objectives

The purpose of the project reported herein was to develop and test next-generation materials (aggregators) for oil spill mitigation. The project was divided into four general tasks:

1. Develop materials that function as both a herder and a sorbent in seawater.
2. Test the aggregator in icy water and sub-zero conditions.
3. Conduct in situ burns (small, mid- and large-scale) and laboratory studies.
4. Determine the combined effect of bioremediation and the long-term storage viability of oil-degrading microorganisms on the aggregator.

The task titles and objectives were as follows:

Task 1 – Materials Development: Aggregating and Sorbent Studies in laboratory and mesoscale testing

Chemically or thermally modify sawdust to gain the following properties:

- enhanced aggregating and sorbent effect

- reduced water absorption and increased oil absorption/adsorption
- enhanced buoyancy
- herding

Task 2 – Herding and sorbent studies in icy water

- Determine the effect of presence or formation of ice on sorption, retention of oil, buoyancy.
- Determine whether ice crystallization forms on aggregator.

Task 3 – Burn studies

Demonstrate the compatibility and utility of using herding agents when a cleanup process includes ISB at the

- laboratory scale
- mesocosm scale
- large scale

Task 4 – Viability and stability of microbial reagents

Assess the following:

- the effect of storing cells on the sawdust media and media with and without additives
- whether a sawdust-based matrix promotes biodegradation in a marine setting
- whether priming a bioremediation mix with oil accelerates the rate of biodegradation.

1.2 Report Contents and Organization

Ensuing sections of this report identify a set of key questions that were addressed in this study and provide a synopsis of the methods used for the exploratory and proof-of-concept tests, followed by conclusions and recommendations. Appendix A contains a glossary and Appendix B describes the chemical synthesis and performance of the aggregator. Appendix B has been redacted due to the proprietary or patent pending nature of its content.

2.0 Aggregator

Next-generation materials for oil spill mitigation—"aggregators"—were developed and tested for buoyancy, sorption capability, and ability to support burning under a seawater spill scenario. A number of materials and processes were screened on these characteristics to find the best-performing materials. The following sections discuss how the aggregator was synthesized, how it performed, and the process of down-selection to a final wood species type, mesh size, and treatment method.

2.1 Aggregator Development

Cellulose was selected as the initial substrate due to its ready availability and low cost as a byproduct of agriculture or timber industries, as well as its burnability. To evaluate its potential, PNNL staff developed and tested several different wood types, sizes, and treatment processes.

Cellulose sources
- Douglas fur
- Poplar
- Pine
- Maple
- Rice hull

Size
- 40-60 Mesh (commercially available)
- 20 Mesh (PNNL generated)

Treatment
- Thermal treatment
- Fatty-acid modification

Candidate materials were evaluated using three performance factors: crude oil sorption, seawater sorption, and buoyancy in seawater. During our preliminary development and testing, Douglas fir and poplar wood flour along with rice hulls were thermally treated in an argon atmosphere with the temperature varying from 230°C to 500°C to modify their chemical properties (Figure 2.1). Thermal treatment as a process for aggregator development was an appealing approach because it is a low-cost and relatively simple procedure.

With increasing temperature, the texture and color of the starting wood flour material transformed from a light brown to a dark charcoal color, and the texture of the starting material became similar to charred wood, as expected (Figure 2.1B). The percent yield of the thermally treated wood flour product decreased as the treatment temperature increased (Figure 2.1A). Rice hull had the largest product yield at all temperatures, and Douglas fir and poplar were similar in final % yield (Figure 2.1). At 300°C, approximately 80% of the mass remained for rice hull, compared to ~70 % for Douglas fir and poplar; at the highest thermal temperature (500°C), rice hull yield was 50% of the starting material compared to ~30% for Douglas fir and poplar (Figure 2.1A).

Thermally treated samples were also evaluated for their moisture sorption after treatment. Dry samples were stored in a closed chamber with potassium carbonate (K_2CO_3 at 43.2% relative humidity [RH]) overnight prior to testing. Moisture sorption decreased with temperature (Figure 2.2), suggesting that thermal treatment could increase hydrophobicity (evaluated by buoyancy tests reported in Section 3.2).

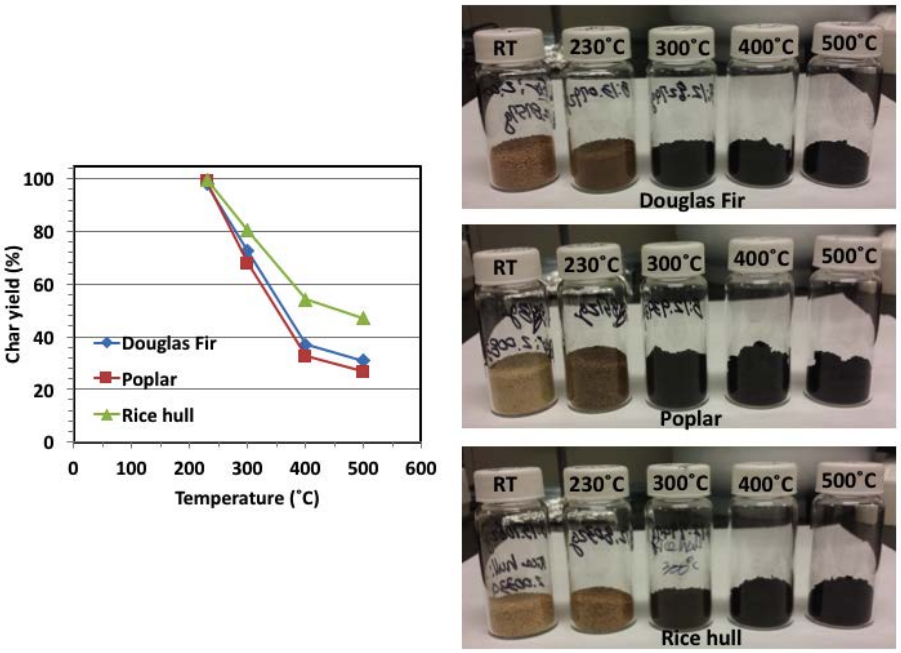

Figure 2.1. Percentage char yield (left) and color change (right) of Douglas fir, poplar, and rice hull as a function of thermal treatment.

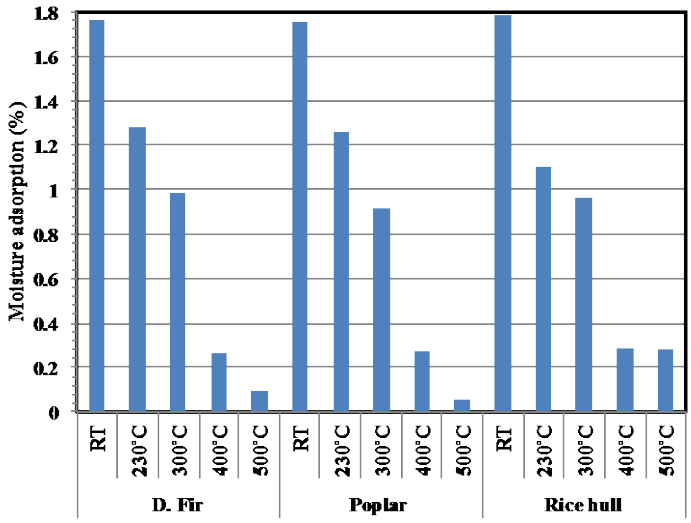

Figure 2.2. Moisture sorption of untreated (stored at room temperature, RT) and thermally treated Douglas fir wood flour, poplar wood flour, and rice hull. Test materials were treated at four different temperatures (230, 300, 400, and 500°C).

The effect on hydrophobicity of attaching fatty acids to the wood flour surface was evaluated. Poplar, maple, pine, and fir wood flours were modified using an acid-catalyzed esterification reaction (Figure 2.3) that bonded oleic acid (OA) to the wood flour. This process increased the size of the wood flour particles (Figure 2.4), total weight (table 2.1), crude oil sorption, and reduced water sorption similar to the 500°C thermal treatment (discussed in section 2.3). A critical difference, however, was that OA treatment resulted in far superior buoyancy compared to thermal treatment: OA-treated sawdust remains buoyant after floating in seawater over several months, whereas 400°C and 500°C thermally treated material

began to sink within the first 10 minutes. This ultimately was the key factor that led to the selection of an OA aggregator as opposed to an aggregator made by the thermal treatment process.

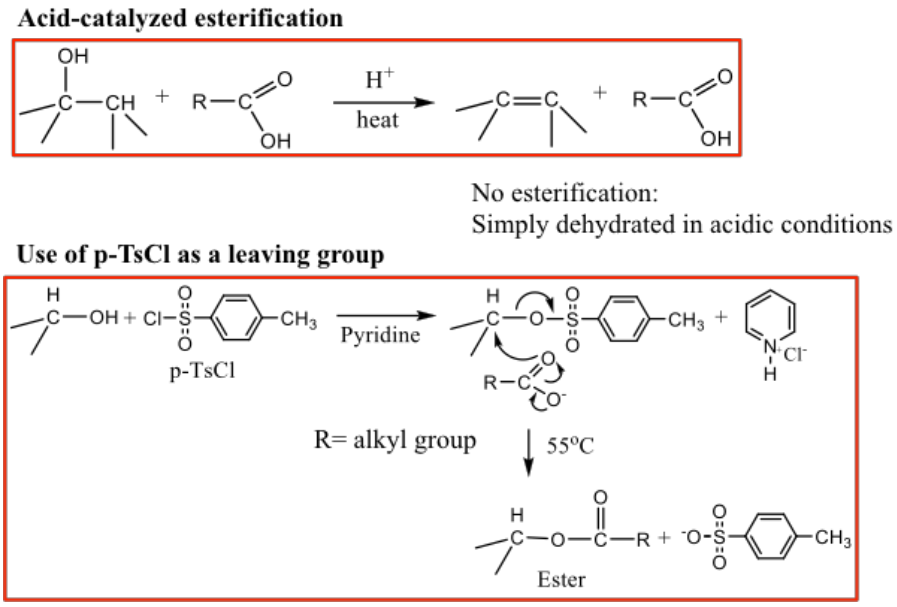

Figure 2.3. Esterification of Fatty Acid. The diagram above illustrates an esterification reaction. By using p-toluensulfonyl chloride as a good leaving group, fatty acid was esterified.

Pine wood flour was found to be the optimal candidate for grafting OA; mesh size minimally contributed to weight percentage gain (WPG; Table 2.1). This work suggests that further chemical modification of the surface that increases the available sites for OA attachment would increase the performance of the aggregator, making for better oil sorption.

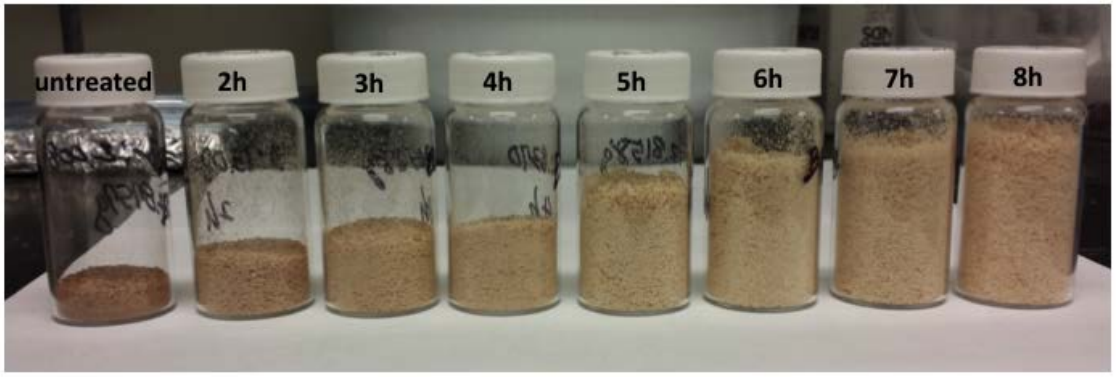

Figure 2.4. Size gain after OA esterification as a function of reaction time. Each vial contained 1 g of Douglas fir wood flour treated with 3.5 g of p-toluensulfonyl chloride, 5 g of oleic acid (OA), and 40 mL of pyridine at 55~58°C..

Table 2.1. Weight Percent Gain (WPG) after Oleic Acid (OA) Modification

Wood flour	Ave. particle size (µm)	Density (g/cm^3)	OA-modification conditions and WPG		
			T (°C)	Reaction time (h)	Max WPG (%)
Maple (20)	850	0.68	55	8	89
Maple (40)	300	0.65	55	8	98
Pine (20)	850	0.51	55	10	165
Pine (40)	300	0.51	55	10	160

Figure 2.5 shows the relative increase of grafted OA as a function of reaction time. Fourier transform infrared (FTIR) spectroscopy analysis showed that OA grafting peaked at ~8 hours (Figure 2.5A). Further experiments that used esterification reactions up to 12 hours (data not shown) determined the extra reaction time yielded only small gains in additional OA attachment to the wood flour substrate. Therefore, an 8-hour reaction time was selected as the optimal reaction time for all subsequent aggregator synthesis.

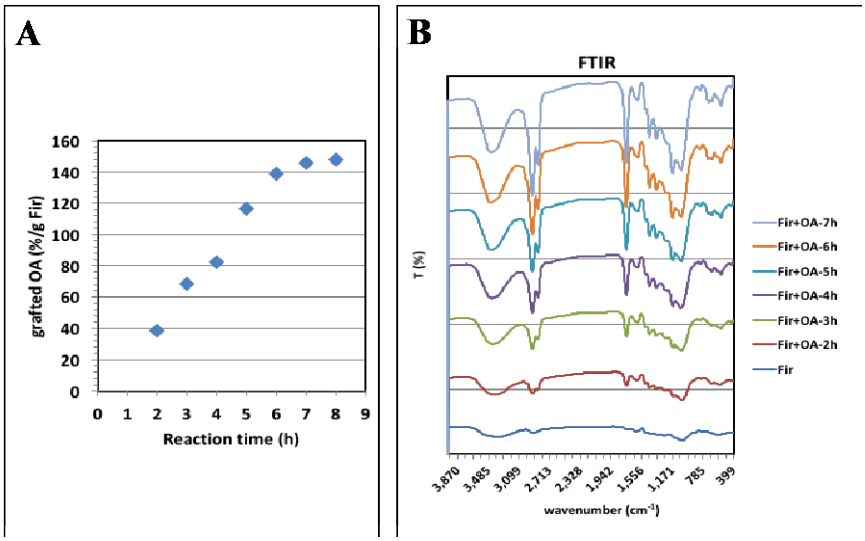

Figure 2.5. Effect of reaction time on the number of OA molecules attached to Douglas fir wood flour (A) and increased esterification as a function of time as confirmed by FTIR (B).

2.2 Buoyancy Test

One of the desired characteristics for an effective aggregator is its ability to float at the surface of seawater and interact with the spilled oil, so the lack of adequate buoyancy in effect results in an ineffective aggregator. Therefore, a series of buoyancy tests were conducted with aggregators synthesized by thermal treatment or OA modification. Test samples (100 mg of Douglas fir wood flour, poplar wood flour, or rice hull) and seawater (10 mL) were placed in a glass vial and shaken for 1 minute. The vials were then placed upright and observed/photographed at fixed time intervals over 24 hours. An unmixed control was also examined. Pictures of thermally treated samples are shown in Figures 2.6 - 2.8. All of the 500°C-treated aggregators displayed very poor buoyancy in seawater, with a significant portion of the material sinking in less than 10 minutes. The 230–300°C-treated aggregators had the best buoyancy, but were still not 100% buoyant. Although the preliminary sorption testing for the thermally made aggregator

was promising; we eliminated this method as a viable option for producing aggregators due to the poor buoyancy.

In contrast, OA-modification of wood flour resulted in an aggregator that was buoyant in seawater for many months and displayed good oil sorption. Figure 2.10 shows OA-treated Douglas fir and poplar aggregator tested in seawater and the organic solvent toluene. The OA aggregator was extremely buoyant in seawater—long-term tests have shown that the aggregator is buoyant for several months. In fact, some of our earlier OA aggregators are still floating in sterile seawater test vials after 15 months.

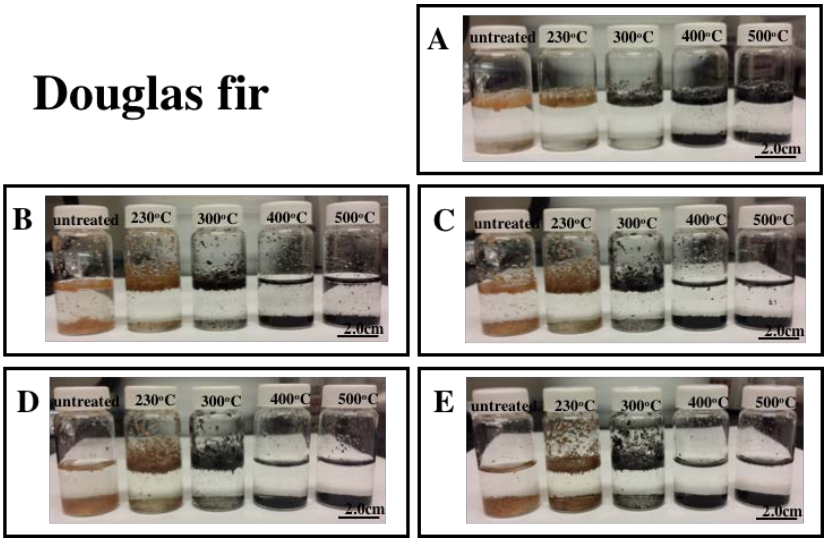

Figure 2.6. Buoyancy of untreated and thermally treated Douglas fir wood flour (A) before mixing, (B) 10 minutes after mixing, (C) 2 hours after mixing, (D) 6 hours after mixing, and (E) 24 hours after mixing.

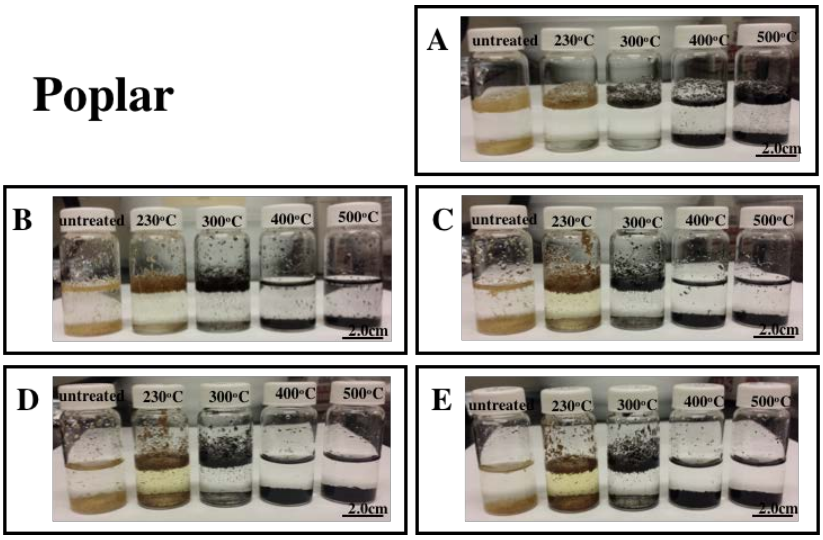

Figure 2.7. Buoyancy of untreated and thermally treated poplar wood flour (A) before mixing, (B) 10 minutes after mixing, (C) 2 hours after mixing, (D) 6 hours after mixing, and (E) 24 hours after mixing.

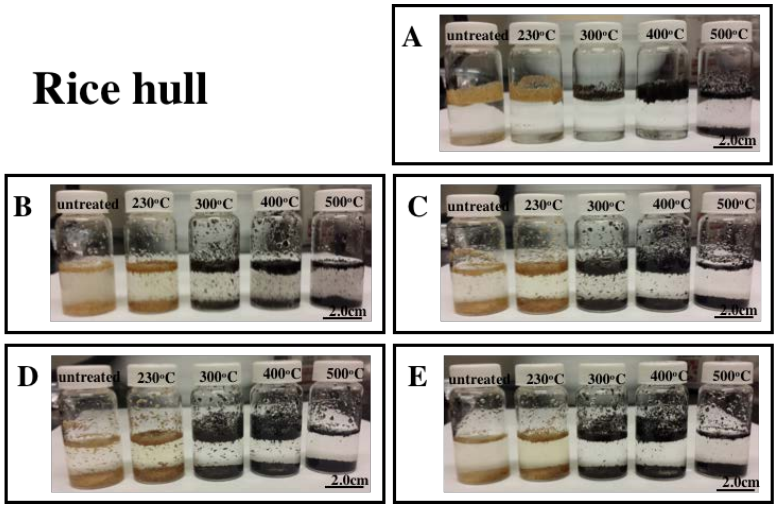

Figure 2.8. Buoyancy of untreated and thermally treated rice hull (A) before mixing, (B) 10 minutes after mixing, (C) 2 hours after mixing, (D) 6 hours after mixing, and (E) 24 hours after mixing.

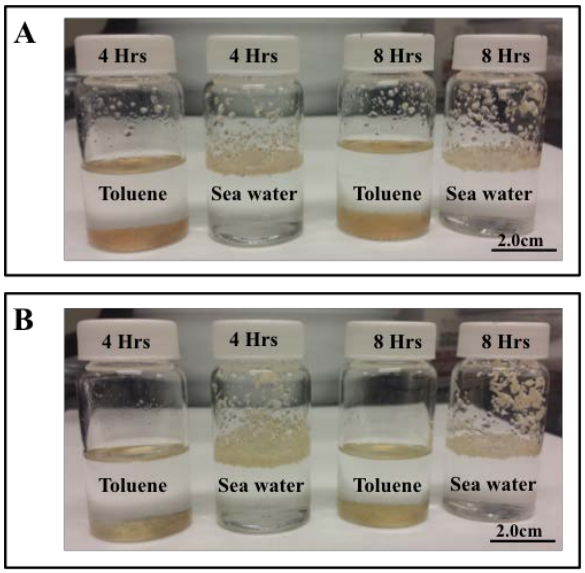

Figure 2.9. Buoyancy of OA-modified Douglas fir (A) or poplar (B) wood flour 4 and 8 hours after mixing in toluene (negative control) and seawater.

2.3 Sorption Testing

Sorption testing for oil and water was conducted for the thermal and fatty-acid aggregators to determine the oleophobicity and hydrophobicity of the aggregators. Moisture sorption was measured by determining the weight percent gains (WPG, [(weight gain/original weight)] x 100) after storing dry samples in a closed chamber with saturated aqueous K_2CO_3 solution (43.2 ± 0.4 relative humidity (RH) at 20^{o}C) overnight.

Oil sorption of aggregator samples was tested with crude oil (West Texas Intermediate). The American Society for Testing and Materials (ASTM) methods for oil sorption capacity measurements (F716-09 and F726-12) were considered suitable for testing the wood flour aggregators because the product stuck to the testing vessel once it was coated with the added crude oil, leading to inconsistencies in mass measurement. Therefore, a new method was developed (Figure 2.10). A premeasured amount of aggregator (~100 mg) was placed in the middle of a 50 mm petri dish. Using a micropipette, crude oil was gradually added to the aggregator until it was saturated, at which point the saturated aggregator was re-weighed to determine the amount of oil that was sorbed by aggregator. Sorption measurements of crude oil were carried out in triplicate for each type of aggregator to get average and standard deviation values.

As the thermal treatment temperature increased, all wood species sorbed more oil and less seawater (Figure 2.11). At 500°C, poplar sorbed the largest amount of oil per mass of product, followed by fir, then rice hull. All wood types sorbed less seawater as the temperature increased. These results were initially promising because thermal treatment is the easiest and most cost-effective of all treatments tested during this project. Unfortunately, the thermally treated aggregators were not buoyant for long periods of time in seawater (see Section 2.2) and they were subsequently removed from consideration for this application.

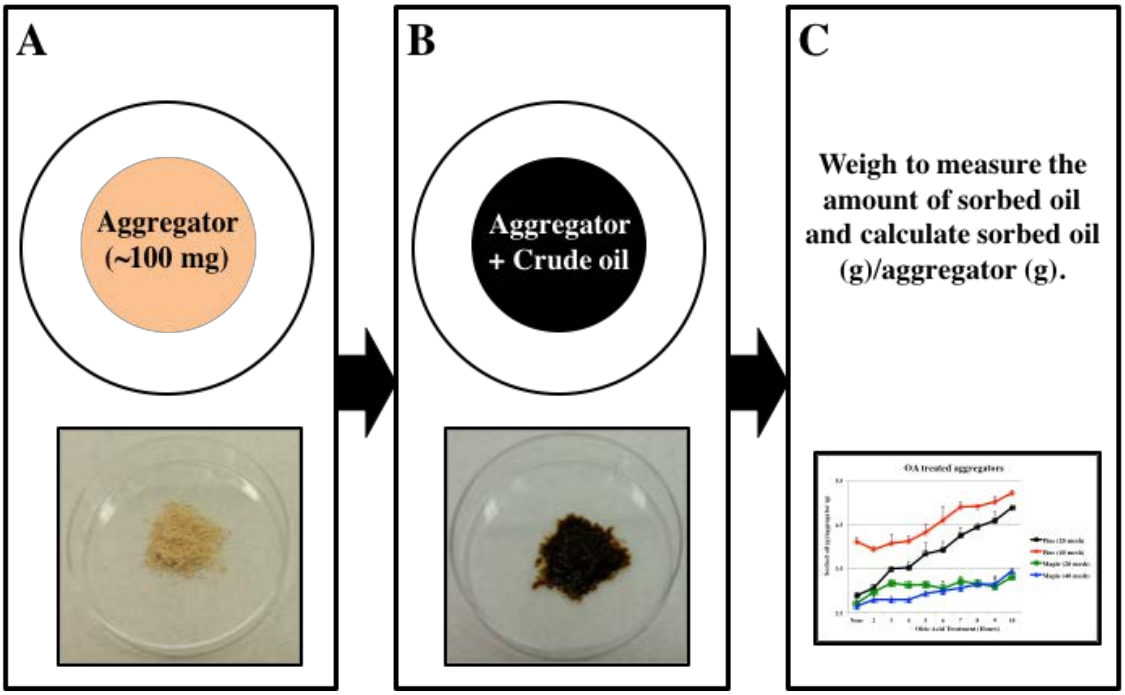

Figure 2.10. Aggregator oil sorption test protocol. (A) Aggregator (~100 mg) was weighed and placed in the middle of a 50 mm petri dish. (B) Crude oil (Texas Raw Crude Oil -TRCO) was gradually added until the aggregator was saturated. (C) The final weight was measured to assess the amount of absorbed oil (g)/aggregator (g).

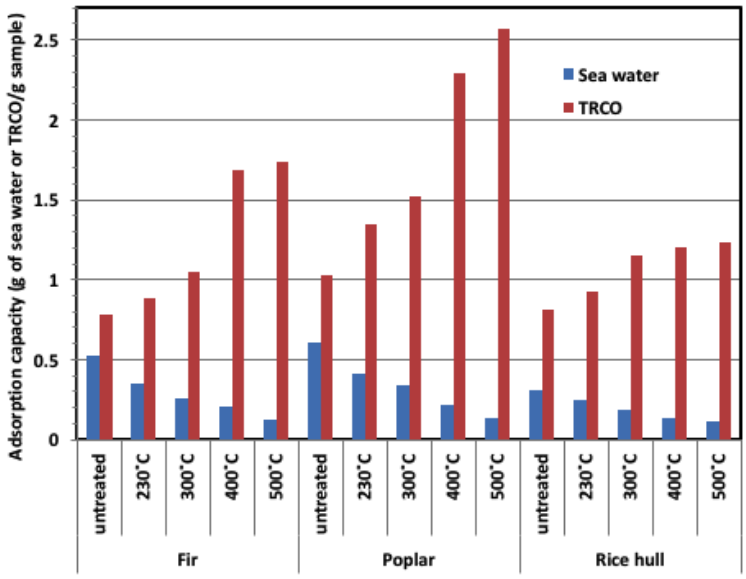

Figure 2.11. Sorption of oil and seawater (measured in grams of seawater or oil per gram of material) by thermally-treated aggregators.

OA modified aggregators were also tested for moisture sorption. Untreated 40-mesh pine wood flour had the highest overall moisture sorption, followed by 40-mesh maple, then 20-mesh pine, and 20-mesh maple. The addition of fatty acid to the surface of the wood flour reduced moisture sorption; this effect was far more dramatic for maple than for pine (Figure 2.12). Only under esterification reactions longer than 7 hours did the pine wood flour lose most of its ability to remove moisture from the air. Thus, it is beneficial, particularly for pine-based OA aggregators, to have a reaction time longer than 7 hours if the intended aggregator will be stored for a period of time before use.

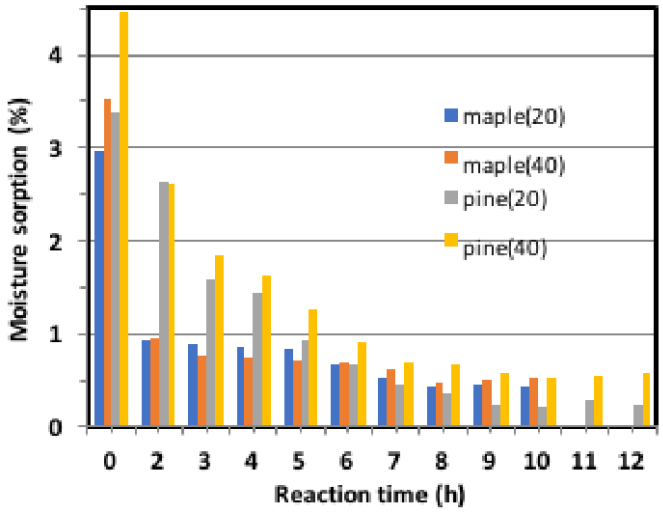

Figure 2.6. Moisture sorption capacity of aggregators as a function of OA-esterification reaction time.

40-mesh pine OA aggregator sorbed the largest amount of crude oil per mass of aggregator product (Figure 2.13). Clearly, the starting wood type and mesh size play a critical role in determining the sorption performance of the aggregator, but the basis for these effects is unknown.

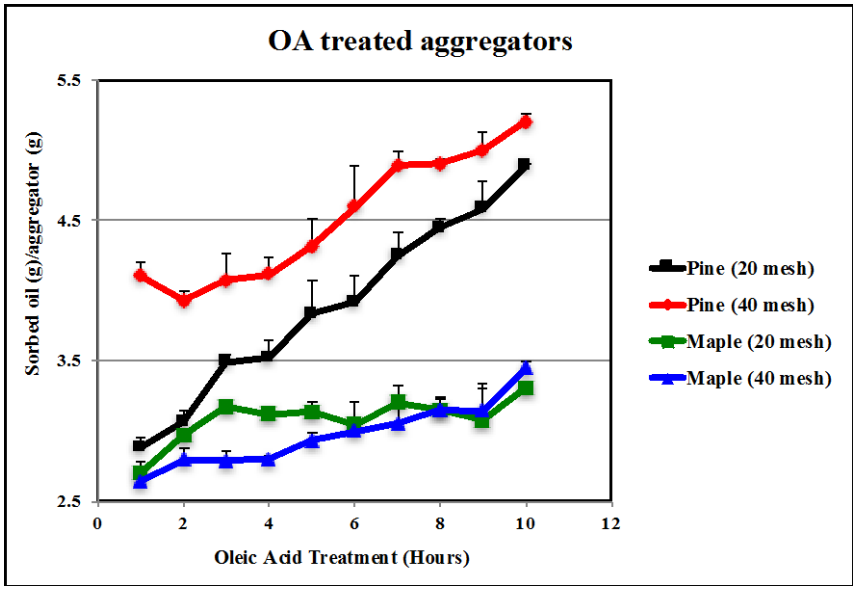

Figure 2.13. Oil sorption by unmodified and OA-treated aggregators as a function of OA-esterification reaction time. Symbols show averages of triplicate tests; bars show standard deviations.

The sorption of oil and seawater by OA-treated pine as a function of esterification time is shown in Figure 2.14. Oil sorption capacity increased roughly linearly with reaction time while the opposite was found for water sorption. Based upon the combination of water and oil sorption results, pine wood flour with a mesh size of 40 and OA-esterification time of at least 8 hours was determined to be the optimal aggregator.

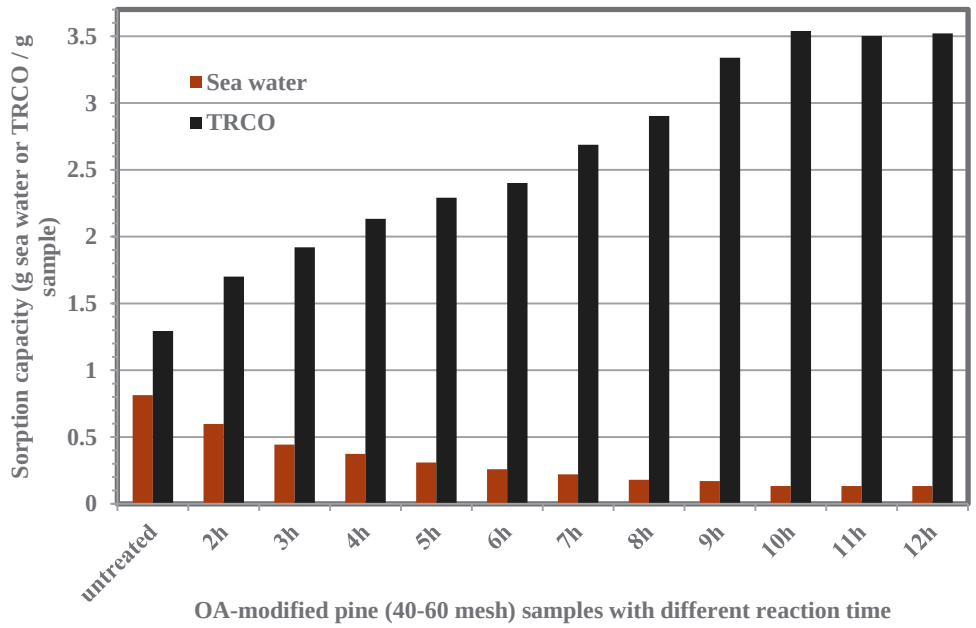

Figure 2.14. Oil (TRCO) and seawater sorption of OA-treated pine wood flour as a function of OA-esterification reaction time.

2.4 Lab-Scale Burn Tests

Lab scale burn tests were conducted to provide a preliminary assessment of aggregator performance and to down-select the newly synthesized aggregator for further testing. PNNL's Marine Sciences Laboratory (MSL) in Sequim, Washington has two burn sites that are currently in use: a small lab-scale burn site that has a 0.5 L crude oil limit, and a mesocosm-scale test area that has a 6.0 L crude oil limit per burn. The small-scale burn site was used for screening candidate aggregator materials. The larger burn site provided a better understanding of the actual material performance when dispensed in larger quantities.

PNNL received approval from the Olympic Regency Clean Air Agency (ORCAA) to conduct these tests prior to execution. Additional support was provided by the PNNL Fire Protection Program, which maintains a qualified fire protection engineering staff to support program development and implementation of program requirements, including those of the National Fire Protection Association and the International Code Council.

2.4.1 Laboratory-Scale Burn Test, Buoyancy, and Oil to Aggregator Ratios

Small-scale burns used 50 mL of crude oil and at least 100 mL seawater in circular Pyrex circular glass dishes (diameter: 150 mm, height: 75 mm). The 40-mesh pine aggregator (8hr esterification reaction) was used in all of the burn tests. Aggregator was added at a 20:1, 10:1, and 5:1 oil:aggregator mass ratios. After 2 minutes of exposure, the surface was ignited (Figure 2.15). After burning, the glass dishes were

kept at room temperature and observations were made at 6 and 72 hours post burn to determine if the burned residue was buoyant. The aggregator left a bubbly residue at the surface after the burn. The texture and consistency of this residue was considerably different from the burned residue typically leftover from a crude oil-only burn and inclusion of the aggregator during burning resulted in a tar residue that is more buoyant than tar residue generated from burning oil only.

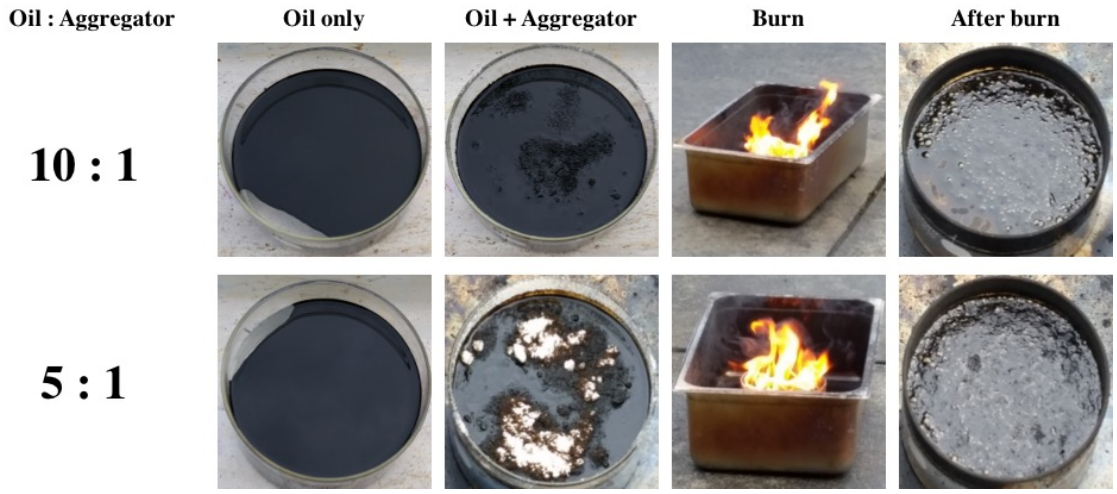

Figure 2.15. Small-scale burn tests.

To simulate the effect of wave action on buoyancy, the burn residues were submitted both to gentle rocking (30 cycles per minute) and strong shaking (200 rpm) (Figure 2.16) over a 72-hour time period. There were no visible signs that the wave action affected the buoyancy of the residue.

The aggregator residue acted like a binding agent with the leftover oil residue, which formed a floating clump. The 20:1 oil:aggregator residue was more loosely bound to the leftover oil than the 10:1 oil:aggregator burn residue. This is apparent in Figure 2.17, because the 10:1 ratio residue formed a tighter clump that floated on the seawater surface in the middle of the test dishes. In comparison, the 20:1 ratio burn residue tended to cling to the side of the beaker. Additionally, at 6 hours post burn, the oil residue of the 20:1 burn covered the entire seawater surface inside the dishes; at the 72-hour time point however, the oily residue had clumped together revealing seawater. Thus, it appeared that residual aggregator remaining after the burn was still acting to sorb unburned oil.

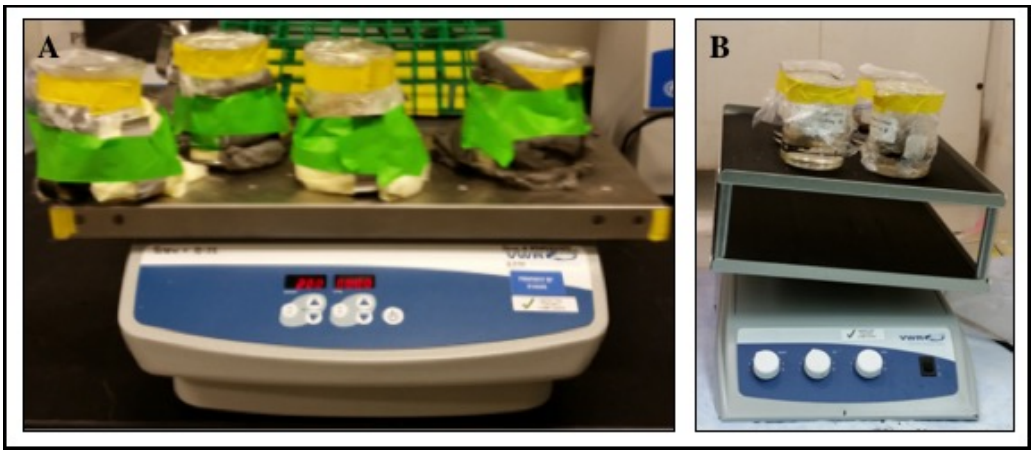

Figure 2.16. Experimental setups for assessing burn residue buoyancy: vigorous shaking (A) or gentle rocking (B).

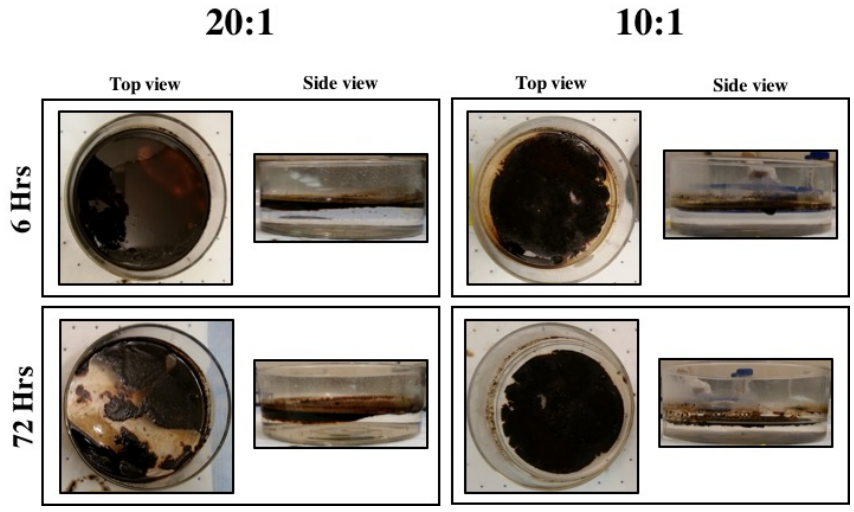

Figure 2.7. Burn residue after 6 and 72 hours post-burn using 20:1 and 10:1 oil:aggregator ratios mixes.

A gentle rocking and high-speed shaking experiment was also performed with residue from an oil-only test burn (Figure 2.18). A considerable amount of oil immediately stuck to the sides of the test beaker at the beginning of the tests. In contrast with the aggregator burn tests, the underlying seawater had a brownish color that was not observed with the oil/aggregator burned residue test. The addition of vigorous shaking caused the oil to disperse farther into the underlying seawater; small oil droplets were dispersed throughout the seawater.

The burned oil/aggregator residue was examined by microscopy in an attempt to determine how much un-burnt aggregator existed in the ash. The residue was examined at magnifications of 40X, 100X, and 400X by light microscopy (Figure 2.19); however, the coloration of the materials made it difficult to determine what percentage of the residue was unburned aggregator. Later analysis using solvent washes (section 3.1) proved to be more successful.

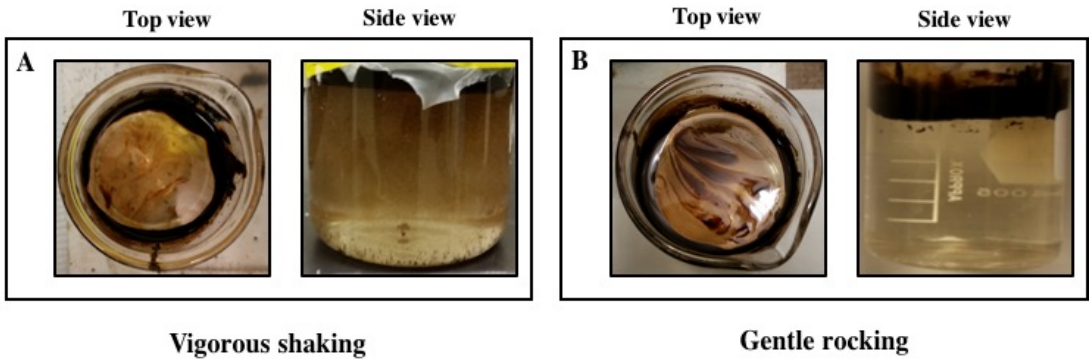

Figure 2.8. Effect of simulated wave action on burn residue generated from oil without aggregator.

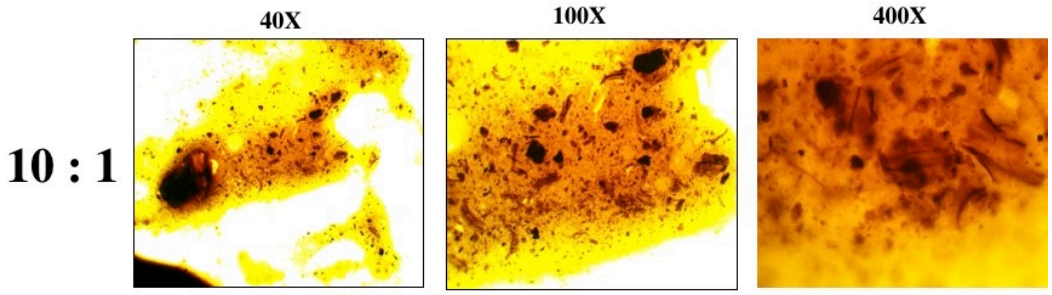

Figure 2.19. Microscopic images of the post-burn residues from a 10:1 oil:aggregator burn.

2.4.2 Large-Scale Test at MSL

Large-scale burn tests were conducted during a week of exceptionally cold weather that formed a ~0.6 cm layer of ice in our 1,135 Liter seawater test tanks each night. Prior to the burn tests, the ice was broken into pieces, then 3.0 L of oil was added. The oil did not spread out over the entirety of the surface, but did spread out to an approximately 1 cm thickness. In the absence of aggregator, the oil ignited and burned very poorly, with a maximum flame height of 60 cm as seen in Figure 2.20 below. However, in the presence of aggregator (14:1 oil:aggregator), an intense burn was achieved with a maximum flame height of 213 cm (Figure 2.21). A third burn with a 50:1 ratio also burned poorly.

A result from the burn tests was that our more efficient burns using the aggregator produced a residue that was very “bubbly.” It was dark amber in appearance and filled with fine bubbles ranging from <1 mm to ~5 mm in size. This tar residue was more buoyant than residual tar from *in situ* burns without aggregator.

Figure 2.20. Large-scale burn of 3.0 L of Dorado crude without aggregator in ice /seawater.

Figure 2.21. Large-scale burn of 3.0 L of Dorado crude with 180.0 g of aggregator in ice /seawater.

2.4.3 Improved Burn Efficiency

We also studied the effect of adding magnesium (Mg) to the aggregator to improve burn temperatures. Mg powder (40–80 mesh) was mixed with the aggregator (1:4, Mg:aggregator by mass). Two sets of test burns were conducted to measure burn temperatures: one using 50 mL and the second using 300 mL of Dorado crude oil. Each set included a burn with crude oil only, oil with aggregator (10:1), and oil with aggregator/Mg mix (10:1). Figure 2.22 shows the small-scale Mg test setup.

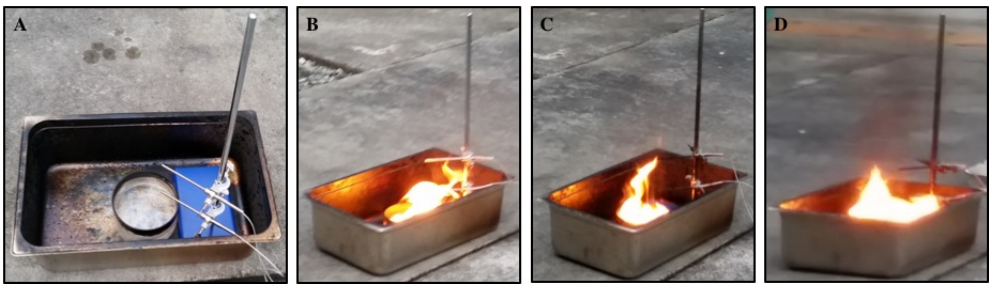

Figure 2.9. Burn test with magnesium; (A) Experiment set up with temperature sensors (B), oil-only burn, (C) oil + aggregator burn, and (D) oil + aggregator + Mg burn.

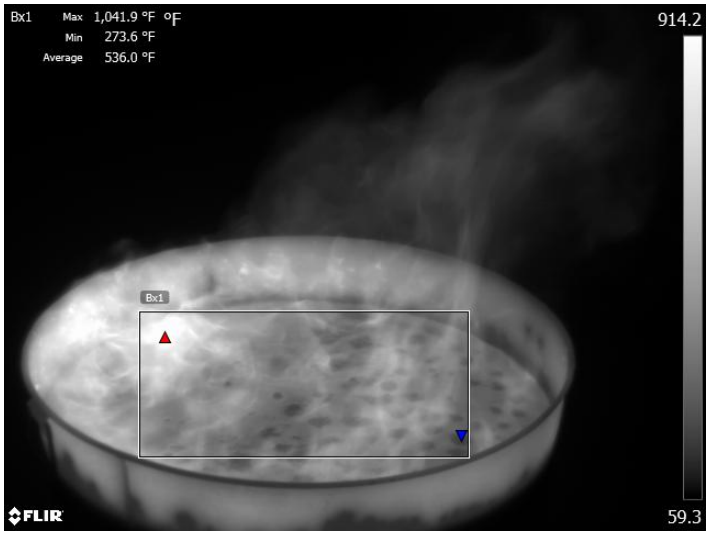

Figure 2.10. FLIR image of burn using Dorado crude only.

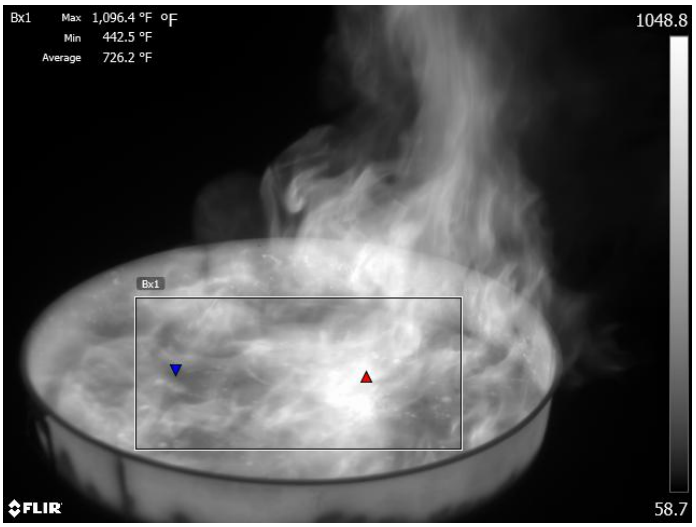

Figure 2.11. FLIR image of burn using Dorado + aggregator.

Figures 2.23 through 2.25 are images of a 300 mL Dorado oil burn taken with a FLIR camera to show maximum and average burn temperatures. The oil-only burn reached a maximum temperature of 1,041.9°F and an average temperature of 536.0°F. The oil + aggregator burn had a maximum temperature of 1096.4°F and an average temperature of 726.2°F. That was a maximum temperature increase of 54.5°F; more impressively, the average burn temperature was increased by 190.2°F. The oil + aggregator + Mg burn had a maximum temperature of 1108.2°F and an average burn temperature of 746.9°F. That was a maximum temperature increase of 66.3°F, and the average burn temperature was increased by 210.9°F. It was clear that the aggregator and aggregator + Mg increased the maximum and average temperatures of a burn at the laboratory scale. However, it was anticipated that the Mg would give a larger maximum temperature than what was recorded. Inspection of the aggregator/Mg burn residue showed that a portion of the added magnesium remained unburned. This could be an artifact of the small-scale burn test that was used; the larger 6 L oil burns may ignite more of the Mg. Reduction of particle size could also support Mg combustion. Further investigation is needed to determine the full potential of the addition of Mg as an accelerant

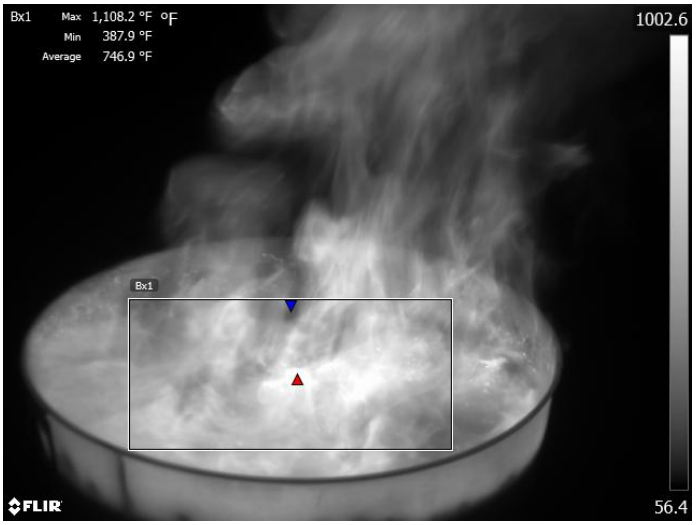

Figure 2.12. FLIR image of burn using Dorado + Aggregator + Mg.

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3.0 Burn Tests at the Joint Maritime Test Facility (JMTF)

A series of burn tests were conducted at JMTF from 10/18/2016 to 10/20/2016 and are summarized in Table 3.1 below. Tests were designed to assess the performance of the OA-modified 40-mesh pine sawdust aggregators at a large scale to better assess the performance of the aggregator product under conditions that better replicate ocean conditions. Two types of crude were tested, Dorado and Alaskan North Slope. Crude oil and aggregator ratios were varied to help identify the best ratio for igniting and supporting/enhancing combustion, sorbing and herding oil.

An oil:aggregator ratio between 10:1 and 6:1 exhibited enhanced combustion; however, the addition of too much aggregator had the effect of suppressing combustion. The tests also demonstrated that a 0.8 mm thick crude oil slick could be ignited with the addition of aggregator to the slick. The aggregator appeared act as a “wick” pulling oil from the seawater surface and maintaining good separation, which allowed for combustion of thinner oil slick thicknesses. The strong “localized” attraction of the aggregator to oil removed the oil sheen from the seawater in several tests. Unfortunately, the aggregator did not result in significant contraction of the slicks (as a herder) during the large-scale tests. Contraction of only a few cm was observed. The presence of the aggregator did not affect oil with which it was not in direct contact. Applied to the perimeter of a slick, it did appear that the aggregator slowed or stopped the continued spread of the oil slick, but this effect was not studied. Further examination is needed to understand how much spreading force (of oil) a given mass of aggregator applied over an area can counteract.

The following photos (are from the burns conducted at JMTF from 10/18/2016 to 10/20/2016. Each picture shows the relative maximum flame height achieved for each crude oil burn; flame height and fire intensity varied as a result of the aggregator/oil ratio being tested. All photos were taken with a GoPro camera. Note: Due to changes in wind direction, the oil boom was placed in different locations in the burn pan over the test period; this was necessary to ensure that facility staff and researchers would not be exposed (downwind) to smoke created by the burn.

The initial burn test B2 (Table 3.1; Figure 3.1) at JMTF used an aggregator:oil ratio of 3:1; the 3:1ratio was the highest concentration of aggregator:oil tested at JMTF. This ratio of aggregator:oil resulted in a portion of the dispensed aggregator not being saturated at the time of ignition (10 minutes after dispensing). The residual non-crude oil-saturated aggregator appeared to suppress the burn, which resulted in observable off-white-colored unburned aggregator after the test burn. Therefore, the remaining test burns used a ratio of less aggregator to oil.

Table 3.1. Burn Data Table from JMTF. The table includes the date of burn, oil type (D = Dorado or A = Alaskan North Slope), density of oil, oil slick thickness, aggregator mass, aggregator:oil ratio, length of burn, approximate flame height, fire temperature, and heat flux. Temperatures listed in table are from mounted thermal couples, not a thermal imaging camera. All volumes listed are approximations based on measured weight. This table and efficiency determination was made by Karen Stone. Modifications made to the table for this report were made by Robert Jeters.

Date/Burn	Oil	Density kg/l	Volume (L/g)	Thick (mm)	Agg (g)	Agg:Oil Ratio	Δt (min:sec)	Flame Height (ft)	Temp °C	Heat Flux (kW/m^2)	Eff?	Residue
10/18 B1	D	852	8.22 L 7504 g	3	0	---	1:35	5	900	45	N	A lot of tar-like residue
10/18 B2	D	852	2.95 L 2670 g	1	964	1:3	3:58	2.5	800	4.75	N	Unburned Agg – no sheen
10/19 B3	D	852	5.90 L 5082 g	2	482	1:11	2:32	4.5	800	9	Y	No Sheen
10/19 B4	D	852	2.95 l 2570	1	265	1:10	1:09	5	798	4.89	N	Lots of trouble igniting. Tar balls
10/20 B5	D*	852	4.84 L 4126 g	1.5	413	1:10	3:27	--	850	18	--	½ g diesel to prime
10/20 B6	A	859	8.22 l 7003 g	3	699	1:6	2:21	10	800	18	Y	Sticky tar. Small amount of unburned oil.
10/20 B7	A	859	8.22 L 7060 g	3	350	1:13	2:37	9	900	15	Y	Sticky tar. Small amount of unburned oil

Figure 3.1. Dorado/Aggregator 3:1 Ratio Burn (B2) at JMTF. The picture is from burn two (B2). A 3:1 ratio of Dorado crude oil and PNNL aggregator were used in this burn test. Image was taken using a GoPro camera.

The thermal image below (Figure 3.2) shows the maximum temperature of burn B2 at 1072°F. This equates to 578°C, which is significantly less than the thermocouple measured temperature. It appeared that the beginning of the thermal video sent to PNNL was missing, so it could not be confirmed by thermal analysis that this was the correct maximum value. In addition to the lower temperatures, burn B2 also had the lowest measured heat flux and flame height of all burns tested with aggregator.

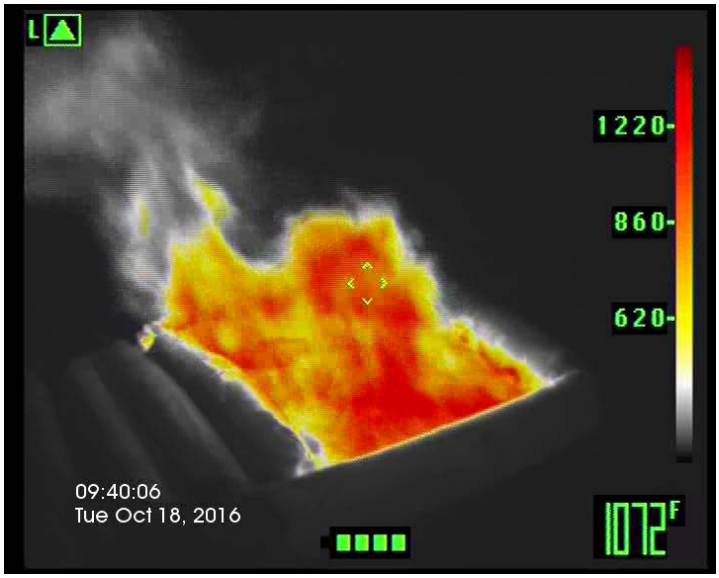

Figure 3.2. Thermal Image of Dorado/Aggregator 3:1 Ratio Burn (B2) at JMTF. The thermal image is from burn two (B2). A 3:1 ratio of Dorado crude oil and PNNL aggregator were used in this burn test. The maximum temperature recorded by the thermal imaging camera was 1072°F.

Figure 3.3. Dorado/Aggregator 11:1 Ratio Burn (B3) at JMTF. The picture is from burn three (B3). A 11:1 ratio of Dorado crude oil and PNNL aggregator were used in this burn test.

Thermal image analysis of burn B3 (Figure 3.4) showed a maximum temperature of 1538°F or 837°C, this corresponds to the thermocouple measured temperature of 800°C (see Table 3.1).

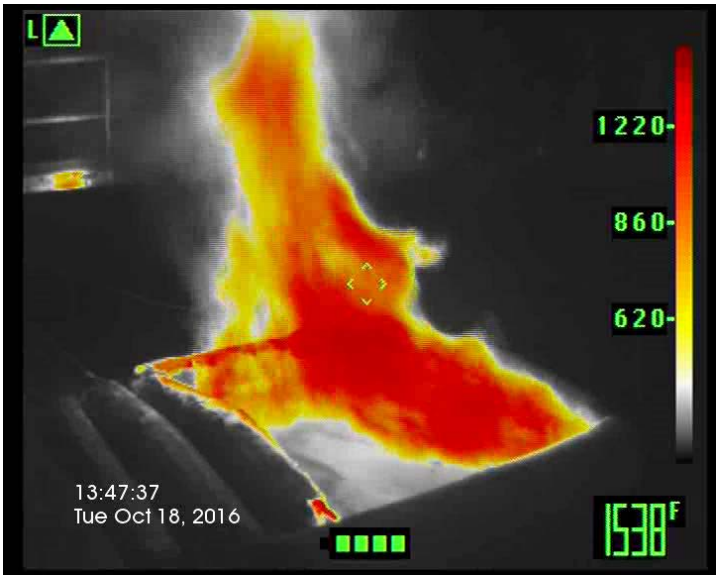

Figure 3.4. Thermal Image of Dorado/Aggregator 11:1 Ratio Burn (B3) at JMTF. A 11:1 ratio of Dorado crude oil and PNNL aggregator were used in this burn test. The maximum temperature recorded by the thermal imaging camera was 1538°F.

Figure 3.5. Dorado/Aggregator 10:1 Ratio Burn (B4) at JMTF. A 10:1 ratio of Dorado crude oil and PNNL aggregator were used in this burn test.

Thermal image analysis of burn B4 (Figure 3.6) showed a maximum temperature of 1529°F or 832°C, this corresponded to the thermocouple measured temperature of 798°C (see Table 3.1). Burn tests B3 and B4 used a similar ratio of oil to aggregator of 11:1 and 10:1, however the initial oil slick depth differed, B3 was 2 mm in initial thickness, whereas B4 had initial thickness of 1 mm. Both burns had similar maximum burn heights and temperatures, but burn B4 was more difficult to ignite with its 1 mm oil thickness. This was to be expected because 1 mm thick oil slicks normally do not ignite unless an accelerant is added. However, ignition was achieved with the addition of the aggregator.

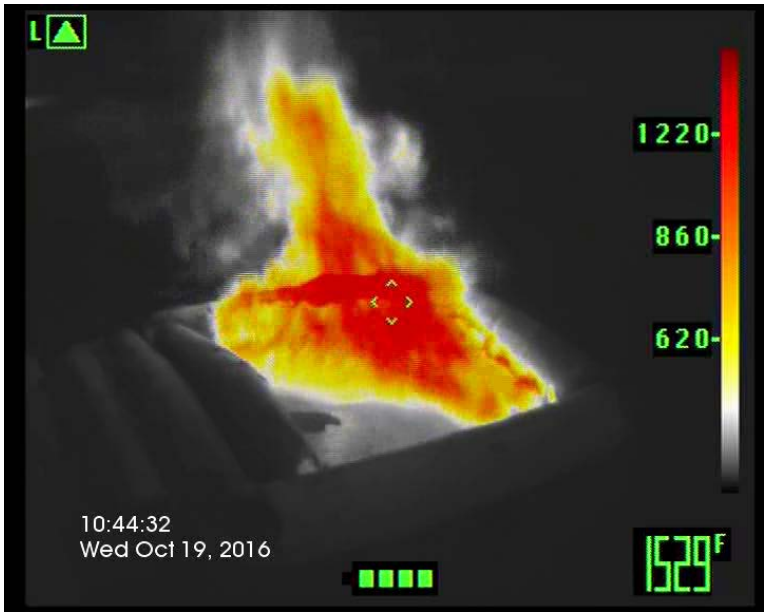

Figure 3.6. Thermal Image of Dorado/Aggregator 10:1 Ratio Burn (B4) at JMTF. A 10:1 ratio of Dorado crude oil and PNNL aggregator were used in this burn test. The maximum temperature recorded by the thermal imaging camera was 1529°F.

Burn B5 (Figure 3.7) tested the use of aggregator on weathered Dorado crude oil. A 10:1 oil to aggregator ratio was used on a slick of 1.5 mm thickness. Unfortunately, the weathered oil proved difficult to ignite; only a few small flames were observed and they quickly extinguished. Crude oil was eventually ignited after the addition of diesel fuel was added.

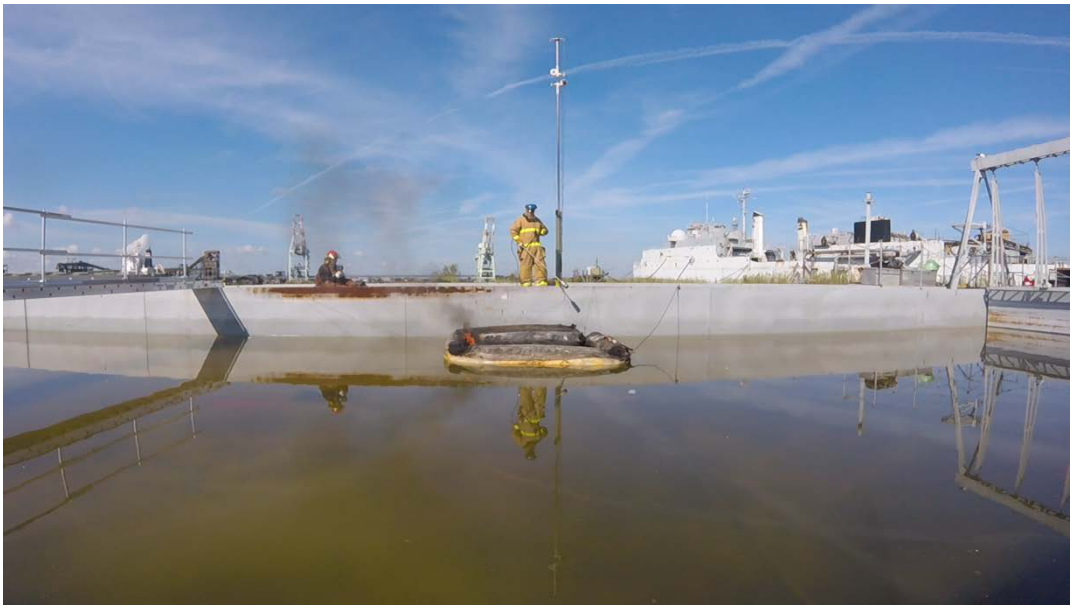

Figure 3.7. Dorado/Aggregator 10:1 Ratio Burn (B5) at JMTF. The picture is from burn five (B5). A 10:1 ratio of Dorado crude oil and PNNL aggregator were used in this burn test. Crude oil was placed on water and left overnight to weather oil.

Burns B6 (6:1 ratio) and B7 (13:1 ratio) (Figures 3.8 through 3.11) produced the most intense aggregator/oil burns at JMTF. The B6 burn reached a maximum temperature of 800°C (Table 3.1) via thermocouple and 910°C (1670°F) by a thermal imaging camera; burn B7 reached a maximum temperature of 900°C (Table 3.1) via thermocouple and 929°C (1704°F) recorded by TIC.

Figure 3.8. Alaskan North Slope (ANS)/Aggregator 6:1 Ratio Burn (B6) at JMTF. A 6:1 ratio of ANS crude oil and PNNL aggregator were used in this burn test.

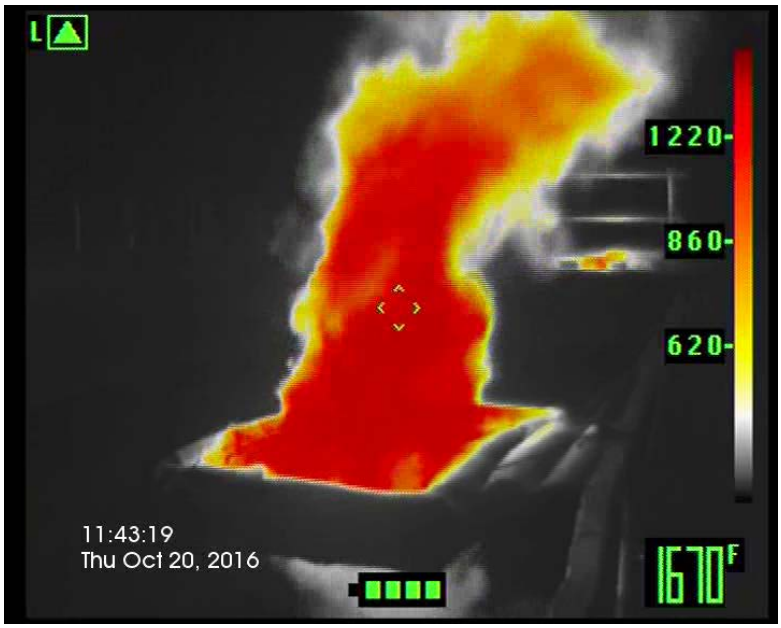

Figure 3.9. Thermal Image of ANS/Aggregator 6:1 Ratio Burn (B6) at JMTF. A 6:1 ratio of ANS crude oil and PNNL aggregator were used in this burn test. The maximum temperature recorded by the thermal imaging camera was 1670°F.

Figure 3.10. ANS/Aggregator 13:1 Ratio Burn (B7) at JMTF. A 13:1 ratio of ANS crude oil and PNNL aggregator were used in this burn test.

Burn efficiency calculations were determined for the Dorado crude oil burn test (B3) and the ANS crude oil burn test (B6). Unburned oil and aggregator for these burns were collected and sent back to PNNL-MSL for post-burn mass determination. The mass of the absorbent pads used in the collection of the burned oil was determined and deducted from the mass of post-burn sample. Pre- and post- weights of the crude oil and aggregator were used to determine the efficiency of burns B3 and B6. Burn B3 had a burn efficiency of 64%,

$$mass\ loss\ during\ burn\ 3582g\ /_{pre\ mass 5564g} \times\ 100 = 64\%\ \text{burn efficiency},$$

burn B6 had a burn efficiency of 72%,

$$mass\ loss\ during\ burn\ 5532g\ /_{pre\ mass 7702g} \times\ 100 = 72\%\ \text{burn efficiency}.$$

Heat flux analysis from the JMTF burns showed that the aggregator has minimal effect on the rate of heat energy transfer for the Dorado crude as tested; the amount of Dorado crude oil was the determinant factor in measured heat flux in all burns at JMTF. Burns B2 and B4 both used 2.95 L of Dorado crude oil, burn B2 used a 3:1 crude oil:aggregator ratio and burn B4 used a crude oil:aggregator ratio of 10:1, yet there was only a heat flux difference of 2.90%. Interestingly, the higher heat flux was from the lower aggregator ratio of 10:1. The larger ratio of aggregator actually suppressed burn B2. When the Dorado crude oil volume was increased to 5.90 L for burn B3, the heat flux was effectively doubled, even though the oil:aggregator ratio was similar to burn B4. Heat flux analysis of ANS crude was inconclusive because only two tests of the same starting oil volume were completed due to time constraints. A 6:1 ratio (B6) of Alaskan North Slope (ANS) oil to aggregator had a better burn efficiency than a 13:1 ratio (B7), but further testing at large scale (JMTF) of oil:aggregator ratios is necessary to determine whether this is significant. Current data suggest that heat flux will be minimally affected by aggregator addition.

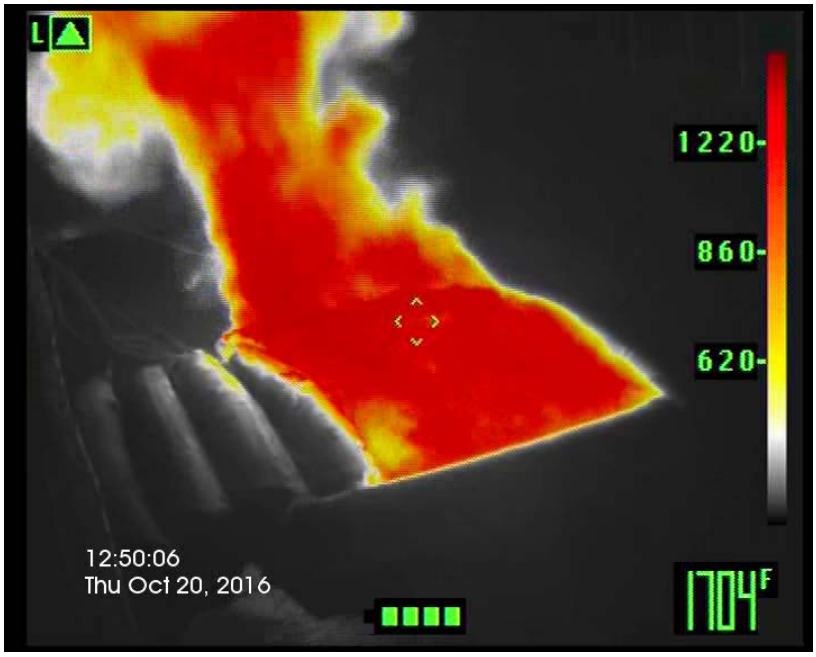

Figure 3.11. Thermal Image of ANS/Aggregator 13:1 Ratio Burn (B7) at JMTF. A 13:1 ratio of ANS crude oil and PNNL aggregator were used in this burn test. The maximum temperature recorded by the thermal imaging camera was 1704°F.

3.1 JMTF Tar Residue Analysis

Post-burn tar residue samples from burn B3 and B6 were collected at JMTF for analysis at PNNL-MSL. Tar residue was analyzed to determine if combustion of aggregator was complete or not after the in situ burn. Tar residue was washed with a solvent to remove tar to determine whether any aggregator remained in the post-burn sample. Gasoline was found to be an effective solvent, because it removed tar and oil from the samples, but did not dissolve the wood flour substrate in the aggregator. An aggregator-only wash was used as a control to determine if the gasoline dissolved or degraded the aggregator. Figure 3.12 shows results from washing. It appears that some of the OA is stripped from the surface of the aggregator, but the wood flour substrate is not effected by the gasoline solvent. This result allowed us to use wood flour as a post solvent wash indicator that aggregator was still present after a crude oil burn.

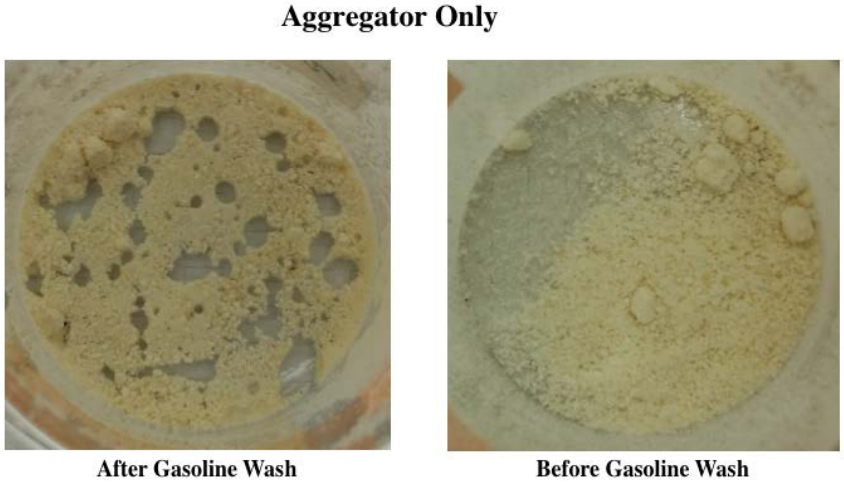

Figure 3.12. Aggregator-Only Before and After Gasoline Wash. The aggregator-only was used as a control to compare with the ANS and Dorado post-burn tar residue samples from the JMTF test burns.

The ANS tar sample (Figure 3.13) had less wood flour present than the Dorado tar sample (Figure 3.14). This is not an unexpected result because the ANS burn (B6) at JMTF has a higher maximum burn temperature of 132°F than the Dorado burn (B3). Thus, the higher temperature achieved in the ANS burn resulted in less residual aggregator post burn. This result is supported by the burn efficiency data that show that the ANS burn (B6) was 8% more efficient than the Dorado burn (B3).

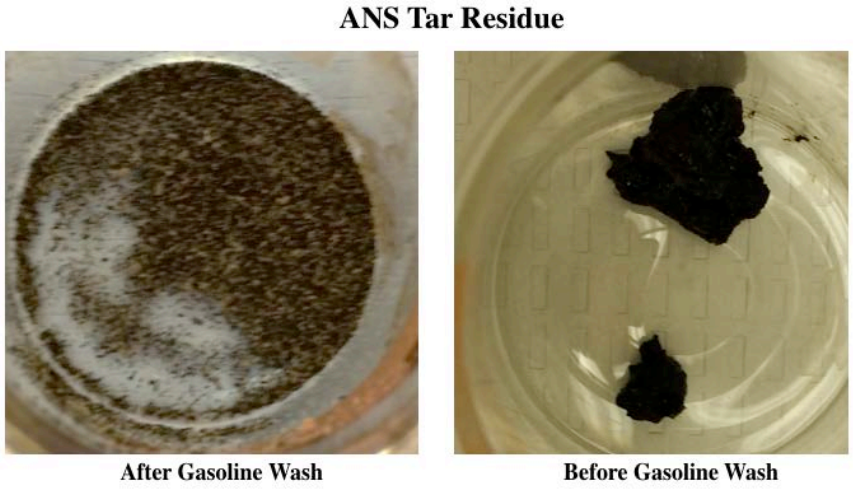

Figure 3.13. ANS Tar Residue Before and After Solvent Extraction. A sample of the ANS tar residue was washed with a gasoline solvent to remove tar and oil residue from the sample to determine if there was any remaining aggregator or wood flour base product remaining in the post-burn sample. The post wash sample showed the presence of a wood product similar to the aggregator-only control.

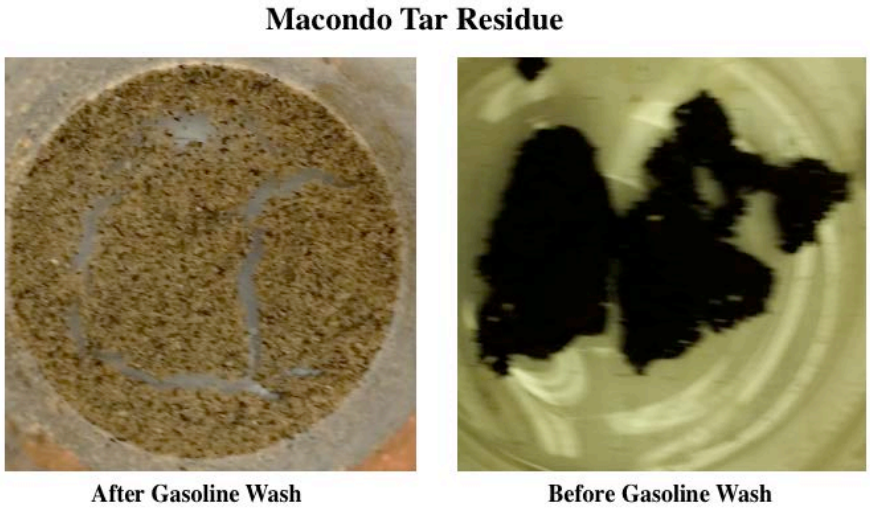

Figure 3.14. Dorado Tar Residue Before and After Solvent Extraction. A sample of the Dorado tar residue was washed with a gasoline solvent to remove tar and oil residue from the sample to determine if there was any remaining aggregator or wood flour base product remaining in the post-burn sample. The post wash sample showed the presence of a wood product similar to the aggregator-only control.

4.0 Viability and Stability of Microbial Reagents

A particular advantage of sawdust is may be used to stabilize and deliver microorganisms for bioremediation for many weeks or longer. Microorganisms can be prepared rapidly in rich media, but after a short period of hours to days waste products produced by the organisms begin to shut down metabolism and kill the cells if they are not separated from that medium. As a consequence, cells typically cannot be prepared in rich medium and stored in advance, and cells prepared at the time of need will begin to die during transport to the point of need. Transferring cells prepared in rich media to sawdust-based materials however, might allow for the long-term storage of viable cells. PNNL evaluated the ability of the aggregator to maintain the viability added oil-degrading microorganisms and any potential impact the aggregator might have on oil bioremediation. Two oil degrading bacteria, *Alcanovorax borkumensis* and *Pseudomonas putida* and one oil degrading fungus, *Hormoconis resinae*, were used for this investigation.

Conceptually, when used to facilitate ISB, some aggregator and sorbed oil could become separated from burn site via wave, current or wind; an infusion of microorganisms into the aggregator might facilitate bioremediation of the escaped oil and this potential effect was evaluated as a means to reduce the impact of the oil. Lignocellulosic material such as wood flour has the potential to keep bacteria and fungi viable for many weeks to months, so the aggregators might serve as a useful vector to introduce bioremediation agents that would accelerate biodegradation. To test the effectiveness of this concept the following objectives were assessed:

- determine how storing cells on the aggregator impacts cell viability
- examine whether adding cells to the aggregator impacts the performance of the aggregator
- determine whether a wood flour-based matrix promotes biodegradation in a marine setting
- determine if priming the cells:aggregator mix with oil accelerates the rate of biodegradation.

Protocol validation and initial testing of bacterial cell suspensions was completed at MSL. Results from growth curves established the time required to reach early stage stationary phase cells of *A. borkumensis* and *P. putida.* Results are described below. The growth curves presented below demonstrate the growth pattern of *A. borkumensis* and *P. putida* over a 3-day trial period using four different dilutions. Validation of these initial tests has allowed us to proceed with our long-term storage testing of these organisms in OA-modified herders.

4.1 Preservation of Oil-Degrading Microorganisms

Long-term storage of oil-degrading microorganisms with the aggregator would enable rapid deployment of the product for ISB and bioremediation, so a series of growth curve experiments were set up to determine the length of time for the bacteria *A. borkumensis* and *P. putida* to reach early stationary phase. In a closed culture system, the stationary phase is a period following active cell growth and reproduction in which cell counts neither increase or decrease, typically due to the depletion of nutrients and buildup of waste products. The stationary phase was chosen because the number of viable bacterial cells would be unlikely to increase during storage with the aggregator, unless the cells were somehow able to metabolize the aggregator. Overnight cultures of *A. borkumensis* and *P. putida* were used to inoculate rich media (marine broth for *A. borkumensis* and nutrient broth for *P. putida*) at four different concentration ratios (overnight culture : fresh media, 1:10, 1:20, 1:50, and 1:100); absorbance was measured at 590 nm at 10-minutes intervals over a 72-hour time period using a Biotek plate reader.

Growth curve experiment results are shown in Figures 4.1 and 4.2. Regardless of the dilution ratios, the length of time required for each bacterium to reach early stationary phases was consistent; 24 hours for *A. borkumensis* and 8 hours for *P. putida*.

To prepare cells for the stationary phase preservation experiments, an overnight bacterial cell culture was used to inoculate fresh medium at a 1:50 ratio. The *A. borkumensis* culture was incubated at 30^{o}C for 24 hours to achieve stationary phase and the faster growing *P. putida* culture was incubated at 30^{o}C for 8 hours to achieve stationary phase. The bacterial cultures (40 mL each) were harvested by centrifugation at 4150 rpm for 5 minutes and washed in cold 1X Phosphate Buffer Saline (PBS). The resulting cell pellet was resuspended in cold 1X PBS to make a cell suspension to inoculate wood flour or aggregator. Spore suspension of *H. resinae* was done by flooding a *H. resinae* plate with sterile 1X PBS, followed by a scrape of the growth on the plate by a cell scraper, and then followed by passing the lysate through a filter of sterile cotton balls. The spore suspension was then diluted in sterile 1X PBS to make a final volume of 40 mL. The final microorganism mix was prepared by combining the two bacterial suspensions and the spore suspension at a 1:1:1 ratio.

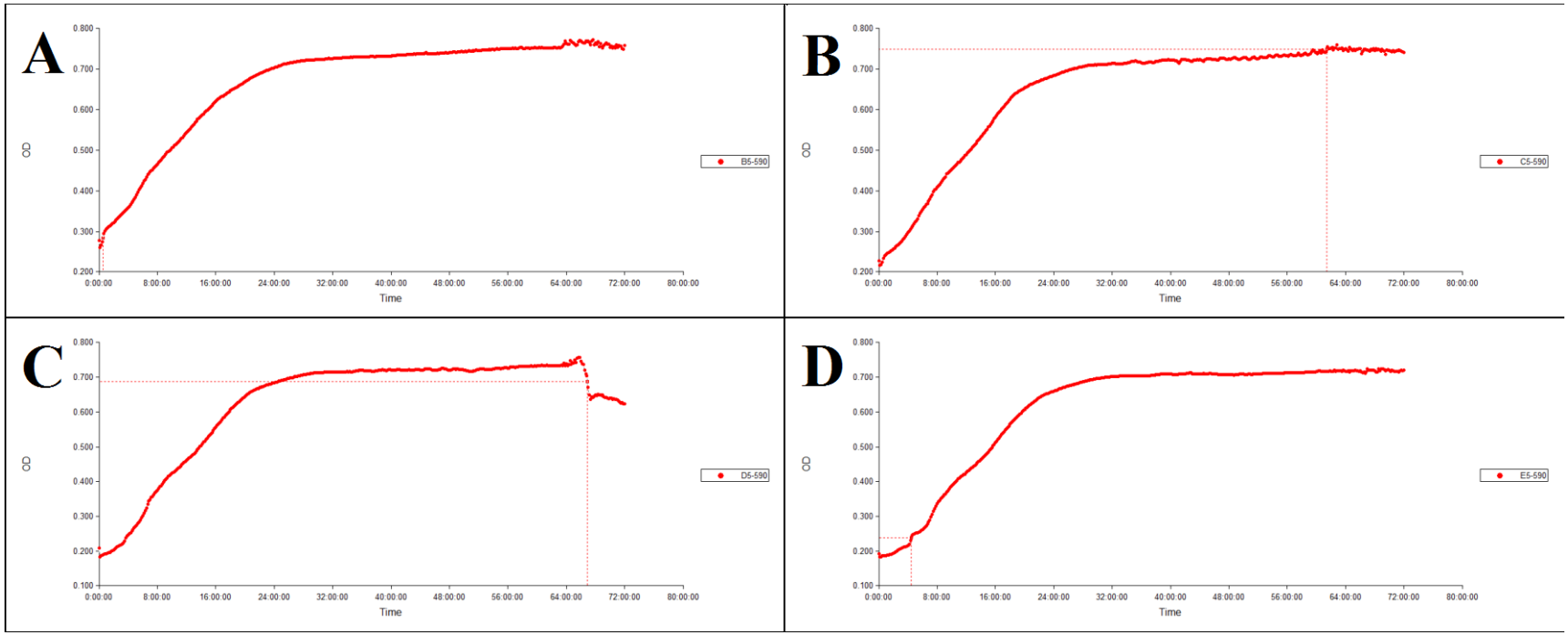

Figure 4.1. *Alcanovorax borkumensis* Growth Curve. Growth curve experiments were done using a 96-well plate and a Biotek Synergy Plate Reader. The culture medium—marine broth—was distributed in a 96-well plate and inoculated with overnight culture (200 µL total volume). Optical film was placed on top of a plate to prevent evaporation and potential contamination among wells. The plate was incubated at 30°C (optimal temperature for growth) and shaken for 15 seconds prior to reading absorbance. Absorbance at 590 nm was measured every 10 minutes for 3 days. Four different dilutions (inoculum:media) were tested 1:10 (A), 1:20 (B), 1:50 (C), and 1:100 (D). X-axis: incubation time (hours), Y-axis: OD_{590}.

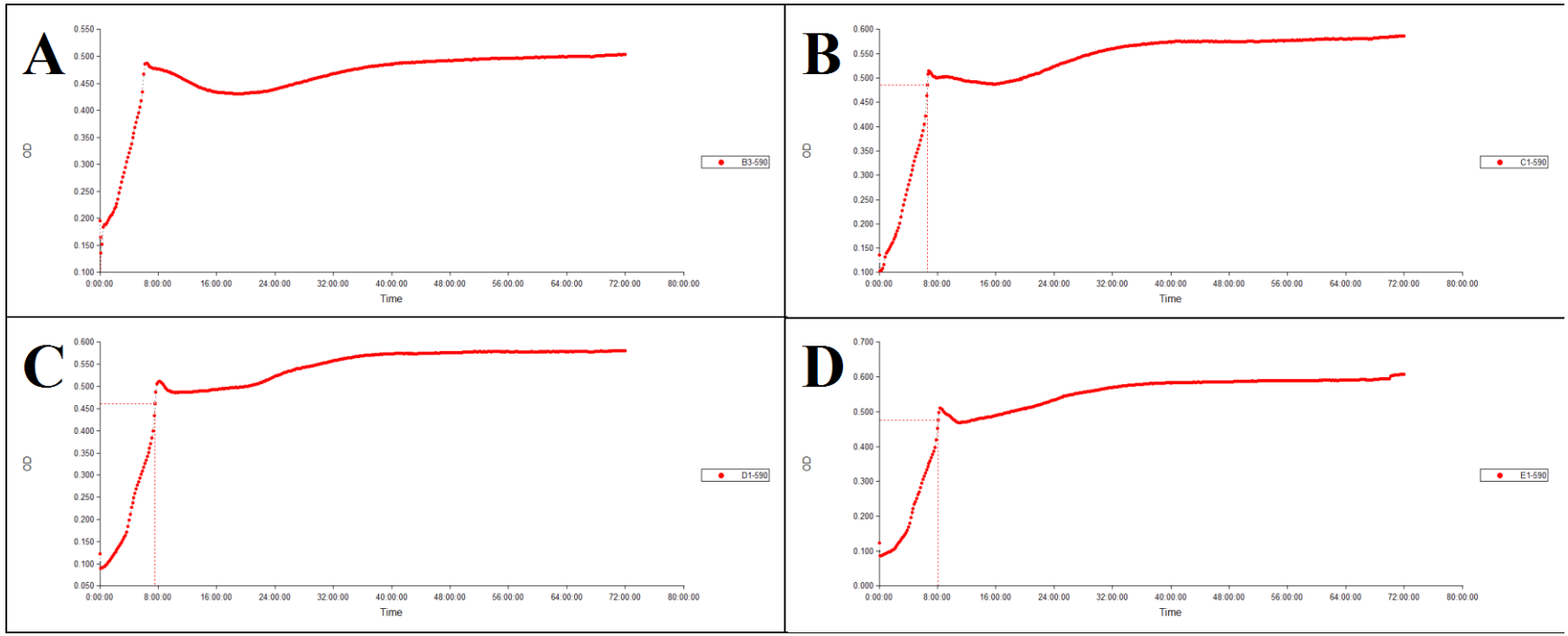

Figure 4.2. *Pseudomonas putida* Growth Curve. Growth curve experiments were done using a 96-well plate and a Biotek Synergy Plate Reader. The culture medium—nutrient broth—was distributed in a 96-well plate and inoculated with overnight culture (200 µL total volume). Optical film was placed on top of a plate to prevent evaporation and potential contamination among wells. The plate was incubated at 30°C (optimal temperature for growth) and shaken for 15 seconds prior to reading absorbance. Absorbance at 590 nm was measured every 10 minutes for 3 days. Four different dilutions (inoculum:media) were tested 1:10 (A), 1:20 (B), 1:50 (C), and 1:100 (D). X-axis: incubation time (hours), Y-axis: OD_{590}.

Prior to performing preservation experiments, unmodified wood flour samples were tested for water sorption. The main goal was to obtain a moist but not too overly wet wood flour and microorganism mixture for easy distribution. Once the microorganisms mix was prepared, it was combined with 2 g of wood flour or aggregator (1 mL for maple wood flour and 1.5 mL for pine wood flour or aggregator). For even mixing, a homogenizer (FastPrep-24^{TM} 5G, MP Biomedicals, LLC.) was used. Settings were as follows; Speed: 4.0 m/sec, Adapter: Tall Prep, Time: 60 seconds.

To manage the large number of samples, a barcode system was developed for efficient sample preparation and data analysis. The barcode system used 39 specification codes as unique identifiers that could be scanned by a standard handheld barcode scanner. A unique barcode was assigned to each test sample to track sample parameters and experimental results. The barcode system was useful in expediting sample preparation by eliminating writing down sample information by hand. Weather-proof labels with a unique barcode and identification number were affixed to all sample vials (Figure 4.3).

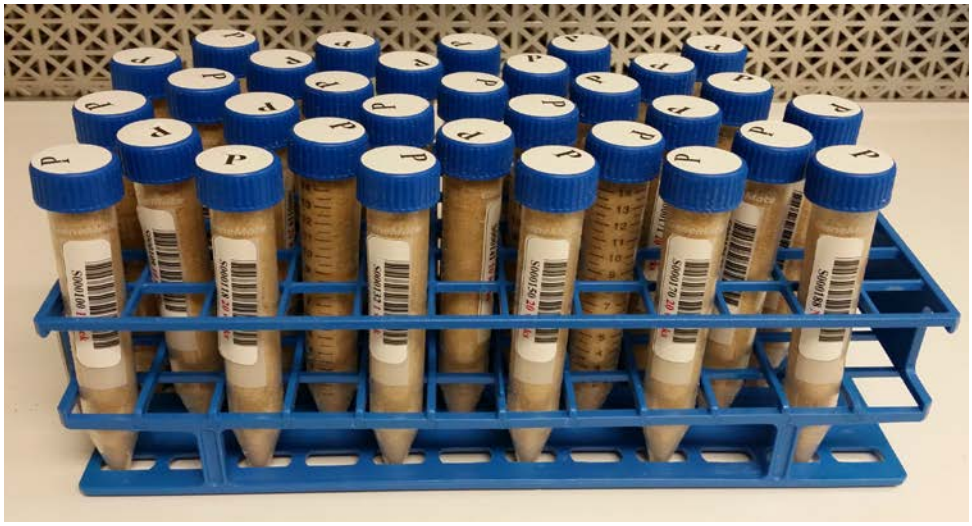

Figure 4.3. Barcode Labels. A unique barcode and ID label was assigned to individual samples. Because two different wood flour samples were tested, tube tops were also labeled (M: maple, P: pine, A: aggregator). In this figure, pine sawdust samples are shown.

The aggregator bioremediation experiments consisted of preservation and biodegradation tests. Figure 4.4 presents an overview of bioremediation experiments.

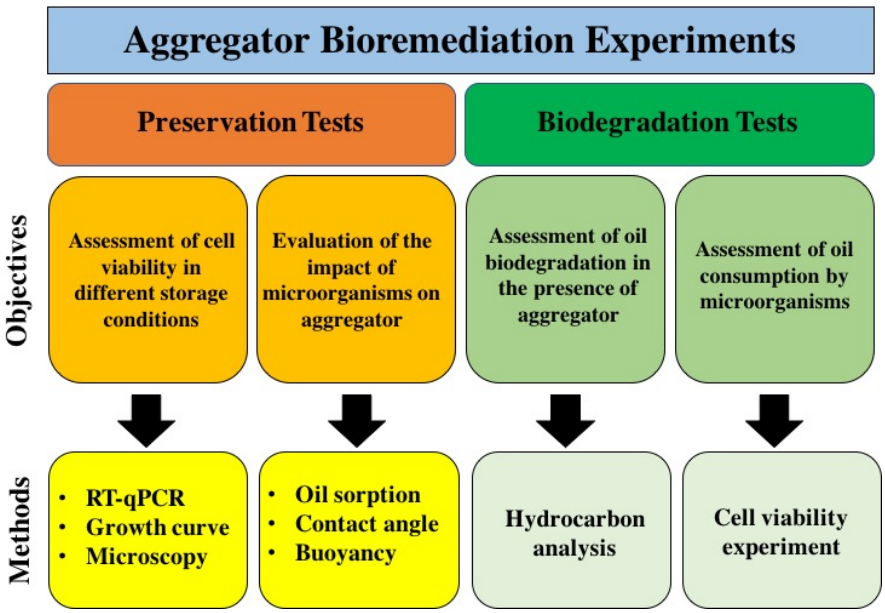

Figure 4.4. Aggregator Bioremediation Experiments (Overview). Bioremediation experiments consist of preservation tests and biodegradation tests. The objectives and methods for the tests are shown.

4.1.1 Preservation Tests

Unmodified wood flour (maple or pine) or OA-treated wood flour (aggregator) was mixed with three representative oil-degrading microorganisms (*Alcanivorax borkumensis*, *Pseudomonas putida* and *Hormoconis resinae*) then stored at three different temperatures; 4°C, 17°C and 30°C. The unmodified wood flour samples were collected and analyzed after 1 week and 8 weeks. Aggregator samples were processed at three different time points: 1 week, 8 weeks and 16 weeks. Two methods were used to assess cell viability: a quantitative reverse transcription polymerase chain reaction (RT-PCR) approach was used to detect and measure metabolic activity and growth curves were used as a direct measure of cell viability. All samples were tested in triplicate.

Samples from each time point were also examined by plain light microscopy to look for obvious signs of growth or changes to the wood substrate. Samples were also assessed for oil sorption, contact angle (hydrophobicity), moisture sorption, and buoyancy.

4.1.1.1 RT-PCR Experiments

At each time point, either the wood flour or aggregator samples were resuspended in cold sterile 1X PBS at a 1:10 ratio wood flour/aggregator:PBS. Total-ribonucleic acid (RNA) was extracted from each resuspended sample or culture using a kit (MegJET RNA kit (Thermo Fisher Scientific, MA, USA) then reverse transcribed to complementary deoxyribonucleic acid (cDNA) then the cDNA was cleaned (ChargeSwitch PCR Clean-up Kit, Invitrogen, CA, USA). Primers for RT-PCR were prepared using Primer 3 software targeting species-specific genes associated with oil degradation. Absolute quantitation standards were prepared by using the prepared RT-PCR primers on the cDNA of pure cultures corresponding to the species-specific primers. The PCR product was cleaned and then quantitated using a NanoDrop spectrophotometer (Thermo Fisher Scientific, MA, USA). The standards were stored at -20°C until needed. Real-time PCR was performed using an ABI 7300 Real-Time PCR System (Thermo Fisher Scientific, MA, USA) and Bio-Rad's iQ SYBR Green Supermix (Bio-Rad, CA, USA). Master mixes

were prepared per the manufacturer's instructions. Samples were arrayed on a 96-well plate in 25 μL reactions; duplicates of no-template controls (NTCs) and three dilutions (1:1, 1:2, 1:10) of standard were also included.

Results from RT-qPCR were analyzed as follows. Mean and standard deviation values of two different NTC end-point fluorescence states were calculated to represent background fluorescence and non-specific amplification. Then the end-point fluorescence value of each sample was compared to the NTC mean to measure active transcription. If the value of a sample was higher than the NTC mean, the sample was determined to be metabolically active (viable).

Figure 4.5 shows the results from cellular metabolism assessment of both unmodified wood flour samples and aggregator stored at 1 week and at 8 weeks. Species-specific primer sets enabled the detection of transcription (metabolically active) cells that were not identified by growth curve and microscopy. After 1 week, most samples showed active transcription, while less activity was observed after 8 weeks (Figure 4.5). *A. borkumensis* may not be an optimal candidate for long-term storage because it became inactive quicker than *P. putida* and *H. resinae* (Figure 4.5). Although the OA aggregator was made with pine wood flour, maple wood flour was also tested to see if hard wood might be better choice to preserve microorganisms long-term. However, pine wood flour was found to be a better choice for preserving organisms for extended time periods. It was found that unmodified pine wood flour was a better substrate to preserve cells than the aggregator (Figure 4.5). The aggregator showed no signs of toxicity to the microorganisms, but surface alteration by the OA modification appears to make the microorganisms less robust in comparison to the wood flour. This might be a side effect of the alteration of the surface chemistry or potential lack of nutrients that exist on the unmodified wood flour.

Maple

	1 Week			8 Weeks		
Temp (°C)	AB	PP	HR	AB	PP	HR
4	+ + +	+ + +	+ +	−	−	+
17	+ + +	+ + +	+ + +	−	+ + +	+ + +
30	+ +	+ + +	+ +	−	+	+ +

Pine

	1 Week			8 Weeks		
Temp (°C)	AB	PP	HR	AB	PP	HR
4	+ + +	+ + +	+ + +	−	+ +	+ + +
17	+ + +	+ + +	+ + +	+	+ + +	+ + +
30	+ +	+ + +	+ + +	+	+ + +	+ + +

Aggregator

	1 Week			8 Weeks		
Temp (°C)	AB	PP	HR	AB	PP	HR
4	+ + +	+ + +	+ + +	+ +	+ + +	+ + +
17	+ + +	+ + +	+ + +	−	+ + +	+ +
30	+	+ +	+ +	−	+ +	−

Figure 4.5. Assessment of cellular metabolism by RT-qPCR (1 week and 8 weeks). For data presentation, the activity of each microorganisms is shown as + or –. Because all samples were tested in triplicate, if all three replicates were active, it is shown as + + +. And if none of the replicate were active, it is shown as –. AB: *Alcanivorax borkumensis*, PP: *Pseudomonas putida*, HR: *Hormoconis resinae.*

At 1 week, the samples subjected to 30°C temperature showed the least number of active cells and after 8 weeks, the temperature effect varied depending on the test materials. In general, 17°C appeared to be the optimal temperature for storage (Figure 4.5). Because pure cultures of these species are normally grown

at 25°C or 30°C, 17°C might be a low enough temperature to suppress the metabolism of microorganisms while keeping them alive.

To compare various factors, three different data presentations for aggregator only results are shown in Figure 4.6.

- Fig. 3A~3C: Cellular transcription activity as a function of storage temperatures
- Fig. 3D~3F: Cellular transcription activity, organized by species
- Fig. 3G~3I: Cellular transcription activity as a function of storage time.

Parameters for the three different data presentations were as follows:

Fig. A~C

- Microorganisms generally stored well at 4°C.
- At 17°C and 30 °C, transcription was not detected in *A. borkumensis* after 1 week.
- Transcription was not detected in any species stored at 30°C for 16 weeks.

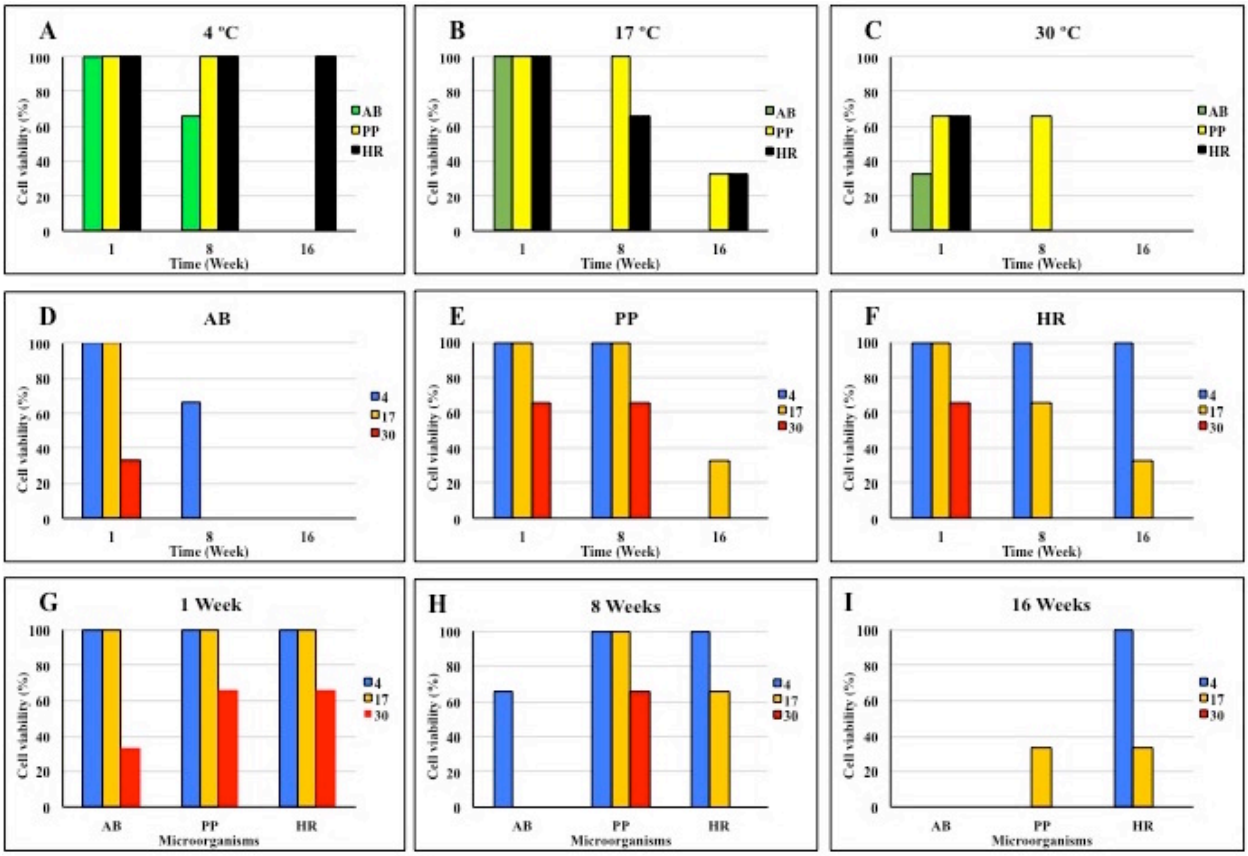

Figure 4.6. Detection of transcription by RT-qPCR after storage at 3 temperatures for 1, 8 and 16 weeks; AB: *Alcanivorax borkumensis*, PP: *Pseudomonas putida*, HR: *Hormoconis resinae.*

Fig. D~F

- *A. borkumensis* remained active on the aggregator up to 8 weeks, but high temperature is not recommended.
- *P. putida* remained active at 4°C and 17 °C especially up to 8 weeks.
- For *H. resinae*, 4 °C was the optimal temperature for storage.

Fig. G~I

- At 30 °C, microorganisms were active only up to 1 week.
- *P. putida* and *H. resinae* were more resilient for long-term storage than *A. borkumensis*.

4.1.1.2 Growth Curve Experiments

To supplement the RT-qPCR data, a second set of experiments was conducted in which growth curves were measured as a direct means of assessing cell viability. Using the samples prepared for the RT-qPCR experiments, at each time point, the wood flour and aggregator samples were suspended in cold sterile 1X PBS at a 1:10 ratio. Then 200 μL of marine broth was placed in a 96-well plate, and 5 μL of the wood flour or aggregator suspension sample was added. Wells containing only marine broth were used as negative controls. The plate was covered with air-permeable optical film to prevent evaporation and potential contamination of or between wells. Using a Biotek Synergy Plate Reader (Biotek, VT, USA), absorbance at 590 nm was measured every 10 minutes for 72 hours. Then the absorbance mean and standard deviation values for medium only control (MOC) were calculated. If the absorbance mean from the sample was higher than the MOC mean, the sample was shown to have viability.

This growth curve method did not seem to be as sensitive as expected and the results (not shown) were inconclusive. This might be because there were not enough viable cells, that recovery in the marine broth was too slow, or the cells remained attached to the substrate during 72-hour growth period that was used.

4.1.1.3 Microscopy

At each time point (time 0, 1 week, 8 weeks, 16 weeks), samples were collected and photographed to determine if the cells made noticeable changes to the appearance of the wood flour or aggregator. Microscopic images were taken at 40X and 100X magnification (AmScope microscope, AmScope, CA, USA). Figure 4.7 shows representative microscopic pictures of two different wood flour and aggregator samples after 8 weeks of storage at 30°C. Although no obvious growth, discoloration, or other effects were apparent, the visual inspection could not be interpreted to mean that the cells had no effect on the products.

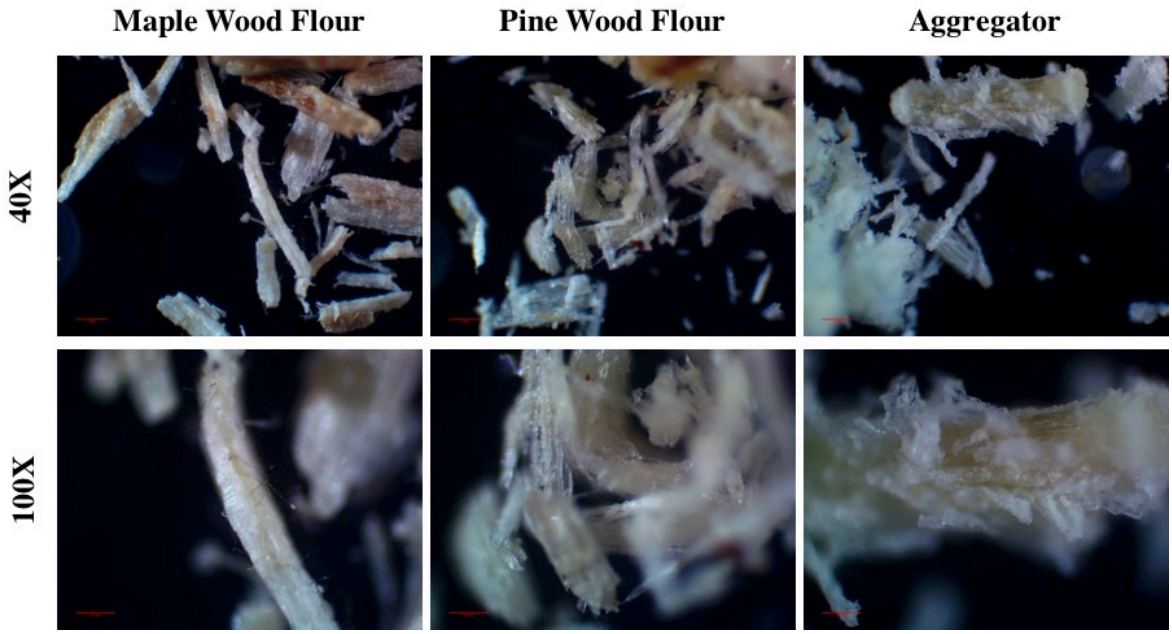

Figure 4.7. Representative microscopic pictures (30°C, 8 Weeks). The maple and pine wood flour samples were mixed with a *H. resinae* spore suspension and the aggregator was mixed with all three oil-degrading microorganisms (*A. borkumensis*, *P. putida* and *H. resinae*).

A series of experiments were then conducted to determine whether the characteristic properties of the aggregator were altered after being mixed and stored with microorganisms. The test evaluated oil sorption, contact angle, and buoyancy at 4°C, 17°C, and 30°C with the microorganism *A. borkumensis*, *P. putida*, and *H. resinae*.

4.1.1.4 Oil Sorption Tests

Figure 4.8 shows results from oil sorption tests with samples collected at four different time points: time 0, 1 week, 8 weeks, and 16 weeks. At time 0, either the unmodified wood flour or aggregator (2 g each) was inoculated with microorganism mix (*A. borkumensis*, *P. putida* and *H. resinae* cell/spore suspension mixed at a 1:1:1 ratio). Materials were stored at three different temperatures (4°C, 17°C and 30°C). Due to moisture derived from the microorganism mix, at time 0, the oil sorption capacity of the aggregator was less than the normal aggregator without the mixture. Higher storage temperature resulted in better oil sorption over time (Figure 4.8). Without the microorganism mix, aggregator oil sorption capacity, sorbed oil(g)/material(g), was 5.45 (standard deviation = 0.16). When aggregator was mixed with microorganisms, sorption capacity was decreased to 3.07 (std dev = 0.13)—about a 44% decrease. After 16 weeks, oil sorption capacity was increased further and 30°C showed the highest recovery—5.22 (std dev = 0.35) sorbed oil(g)/material(g), or about 96% of the regular aggregator oil sorption capacity. At 4°C and 17°C, oil capacity was recovered up to 68% and 82%, respectively. Figures 4.9 through 4.11 show pictures of test materials with and without crude oil.

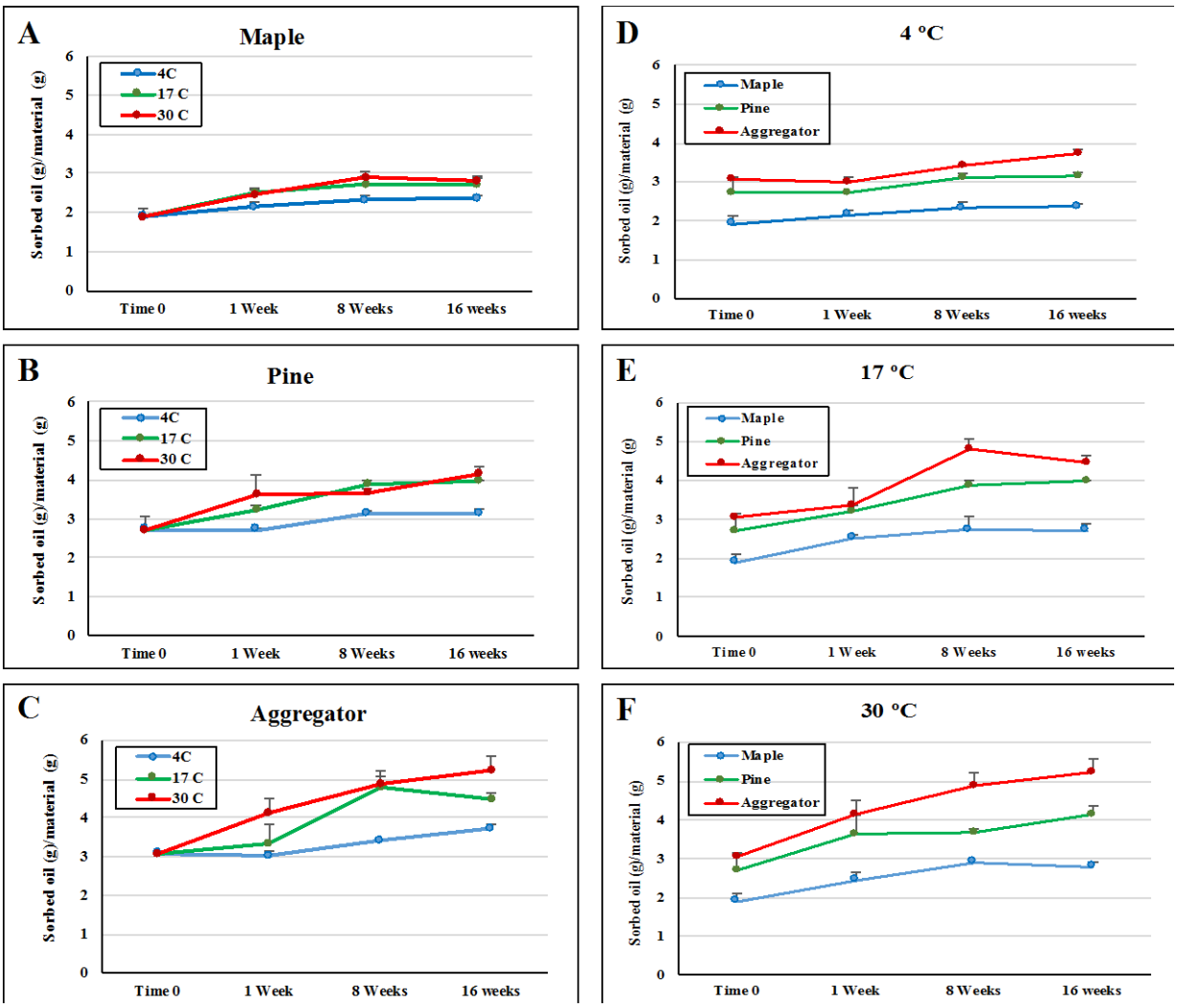

Figure 4.8. Oil sorption test results. (A–C) show oil sorption capacity per material and (D–F) show oil sorption capacity per storage temperature. All experiments were done in triplicate and mean values are shown. Standard deviations are shown as error bars.

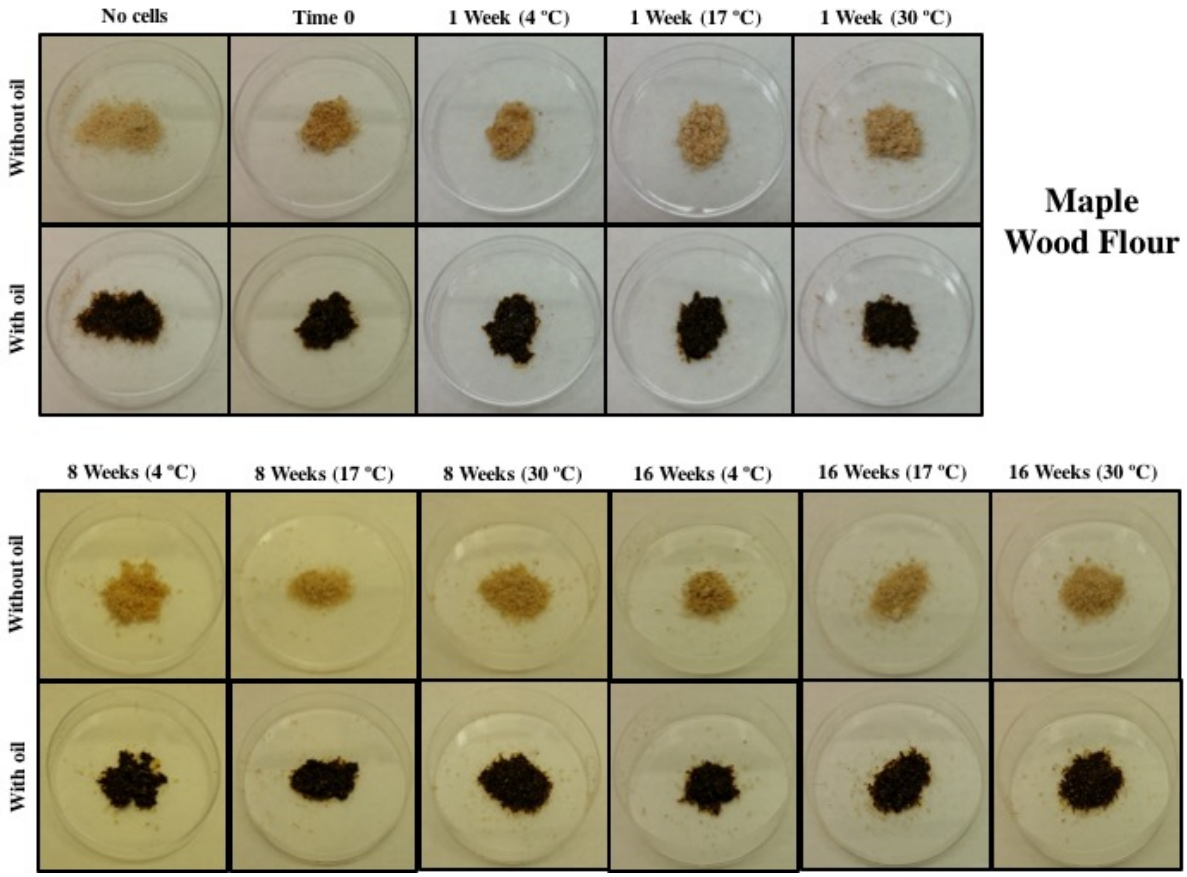

Figure 4.9. Oil sorption tests with maple wood flour (~100 mg each) after being stored at three different temperatures (4°C, 17°C and 30°C) for 16 weeks. Test material was saturated with crude oil (West Texas Intermediate).

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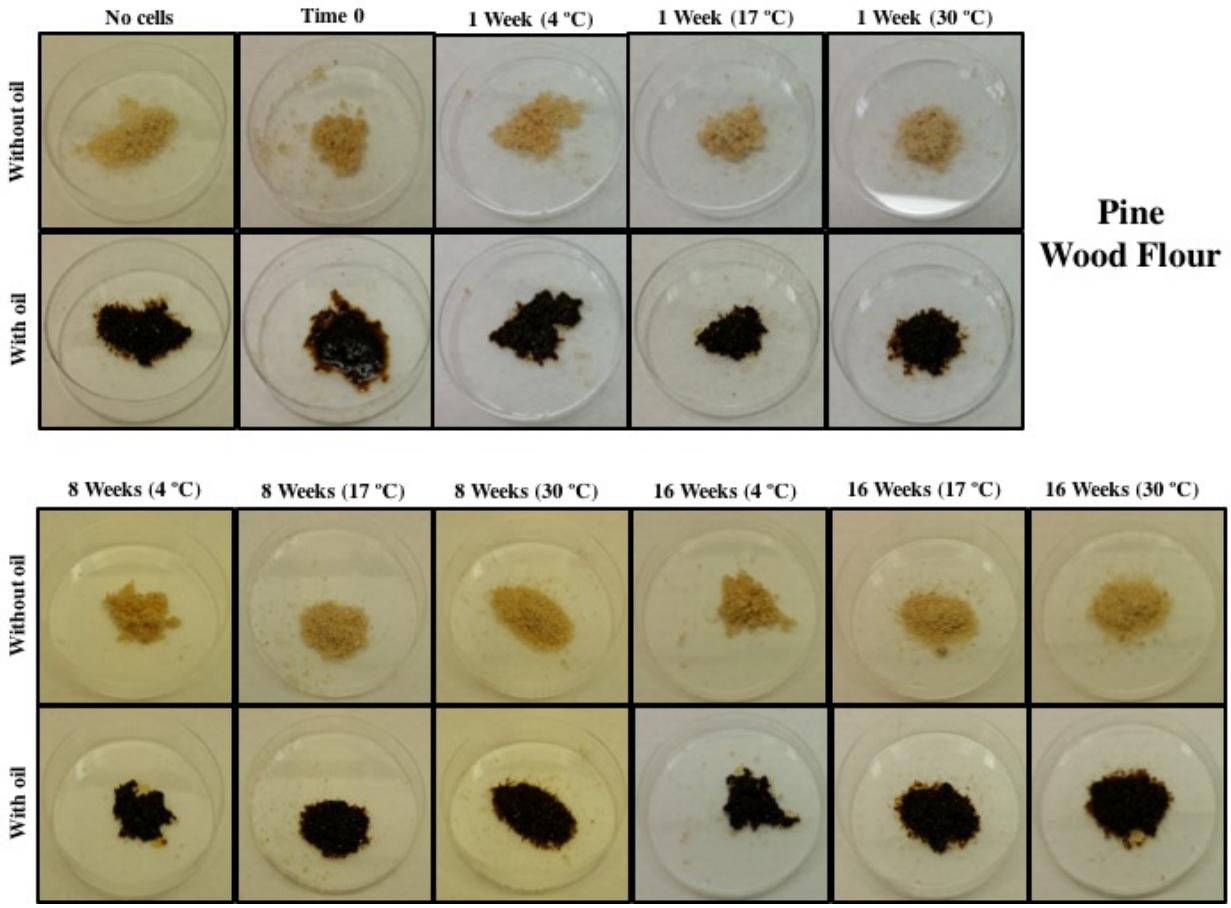

Figure 4.3. Oil sorption tests with pine wood flour (~100 mg each) after being stored at three different temperatures (4°C, 17°C and 30°C) for 16 weeks. Test material was saturated with crude oil (West Texas Intermediate).

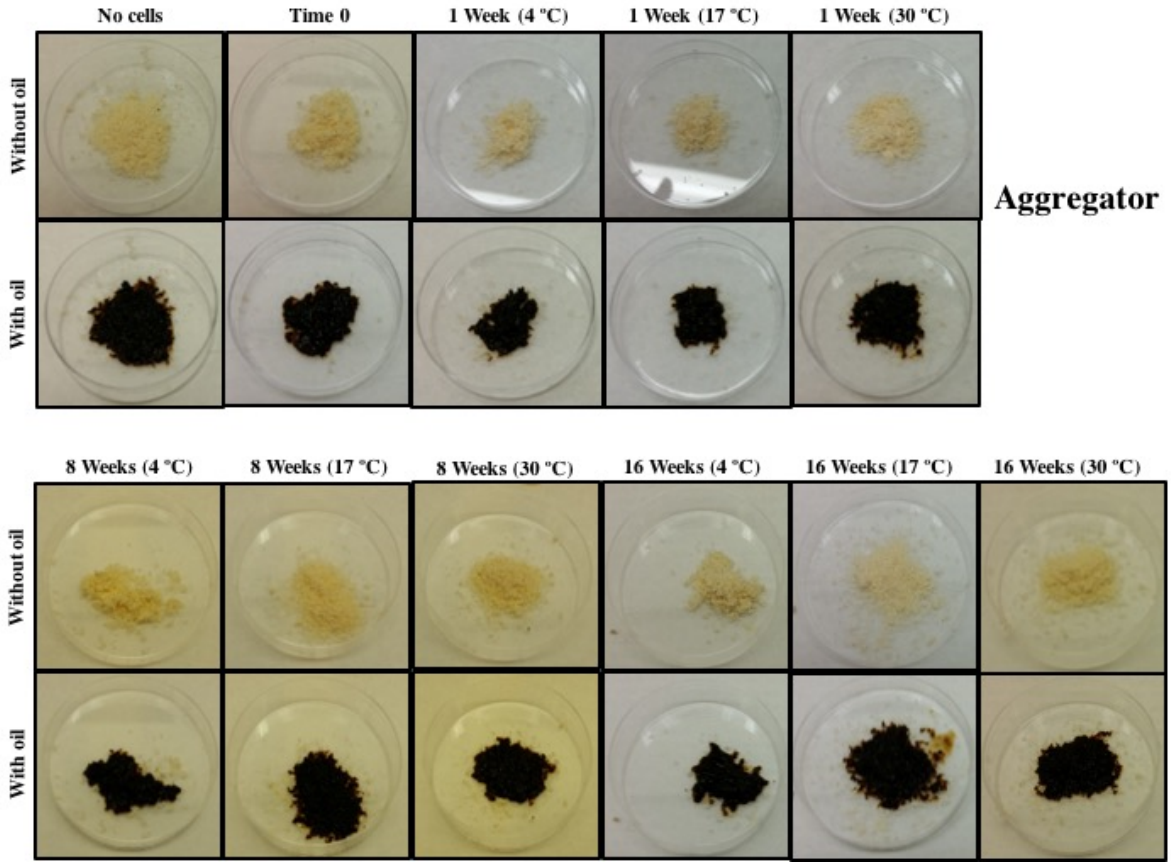

Figure 4.4. Oil sorption tests with aggregator (~100 mg each) after being stored at three different temperatures (4°C, 17°C and 30°C) for 16 weeks. Test material was saturated with crude oil (West Texas Intermediate).

4.1.1.5 Contact Angle and Moisture Content Measurement

The aggregator material is highly hydrophobic and absorbs little seawater compared with unmodified wood flour. These properties are important to sustain, for example, buoyancy, oil sorption, and ice repulsion. To determine if inoculating and storing the aggregator with microorganisms would impact the hydrophobicity of the aggregator, samples were tested for their hydrophobicity by measuring contact angle with filtered seawater using a goniometer (ramé-hart 590-U1 automated goniometer, ramé-hart instrument, NJ, USA). Both aggregator and unmodified wood flour samples were analyzed for comparison. Surfaces with a contact angle >90^{o} are considered hydrophobic or of low wettability (Yuan, Y and Lee TR, 2013). Surfaces with a contact angle >120^{o} are considered highly hydrophobic and those with a contact angle >150^{o} are referred to as superhydrophobic. Unmodified wood flour or aggregator samples were dispensed on a glass microscope slide to which a piece of double-sided tape was affixed. The test materials were gently pressed onto the double-sided tape with a spatula then gently tapped to remove excess material. The slide was placed on the goniometer stage, and a 10 µL drop of 0.45 µm filtered seawater was placed on the surface for contact angle (CA) measurement.

Figure 4.12 (A–C) shows the CA measurement results after 1, 8, and 16 weeks of storage with the microorganism mix (*A. borkumensis*, *P. putida* and *H. resinae* cell/spore suspension mixed at a 1:1:1 ratio) at 3 different temperatures (4°C, 17°C and 30°C). Compared to unmodified wood flour samples, the

aggregator remained hydrophobic throughout the 16 weeks, and there was little impact of storage temperatures on its hydrophobicity (Figure 4.12C).

As previously shown in section 2.3, another property of the aggregator is that it absorbs little water. To determine if inoculation and storage with the microorganisms impacted moisture retention, moisture analysis was done using ~0.25 g of aggregator or wood flour sample and Mark 4 Moisture Analyzer (Sartorius Omnimark, NY, USA). The moisture content was calculated as a percentage of initial weight. As expected, higher temperatures resulted in less moisture content Figure 4.12D).

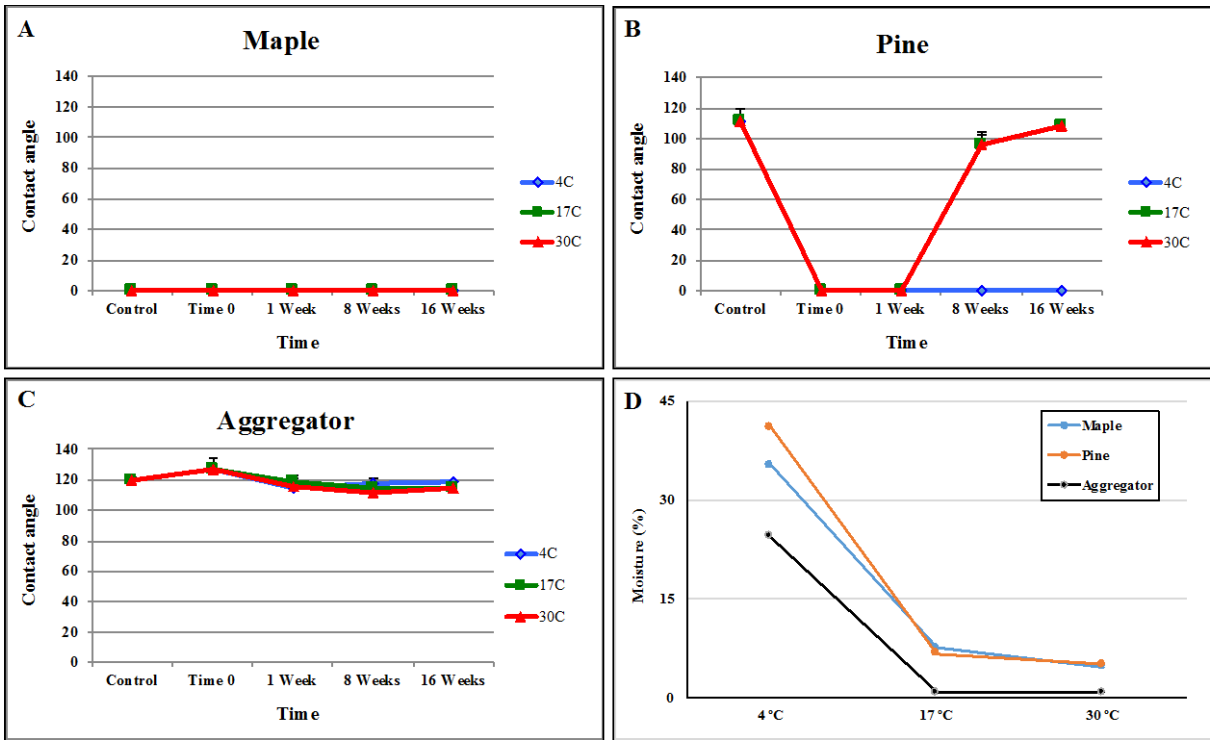

Figure 4.5. Contact angle (CA) and moisture content measurements of control samples (no inoculum) and inoculated samples stored up to 16 weeks at 4°C, 17°C, and 30°C. (A) maple wood flour CA, (B) pine wood flour CA, (C) aggregator CA, (D) moisture content (%) all after 16 weeks of storage at 3 different temperatures.

4.1.1.6 Buoyancy Test

As described previously in section 2.2, buoyancy is another desirable property of the aggregator. To check whether inoculation and storage with the microorganisms might impact buoyancy, unmodified wood flour or aggregator samples mixed with microorganisms were weighed (~100 mg) and placed inside a small vial, then an aliquot of 10 mL of filtered seawater was added. The mixture was vortexed for 1 minute and pictures were taken 30 minutes after mixing.

Figure 4.13 shows the buoyancy test pictures for time 0 samples with or without the microorganism mixture. Adding the microorganism mixture did not have an obvious effect on the materials' buoyancy: the unmodified wood flour samples sank as expected and the majority of the aggregator remained buoyant. Test samples mixed with microorganisms were also stored at three different temperatures and collected at three different time points and tested for their buoyancy (Figure 4.14). These qualitative tests suggest that mixing microorganisms with the aggregator followed by storage at different temperatures and

for different lengths of time does not significantly affect the buoyancy of the aggregator (Figures 4.13 and 4.14).

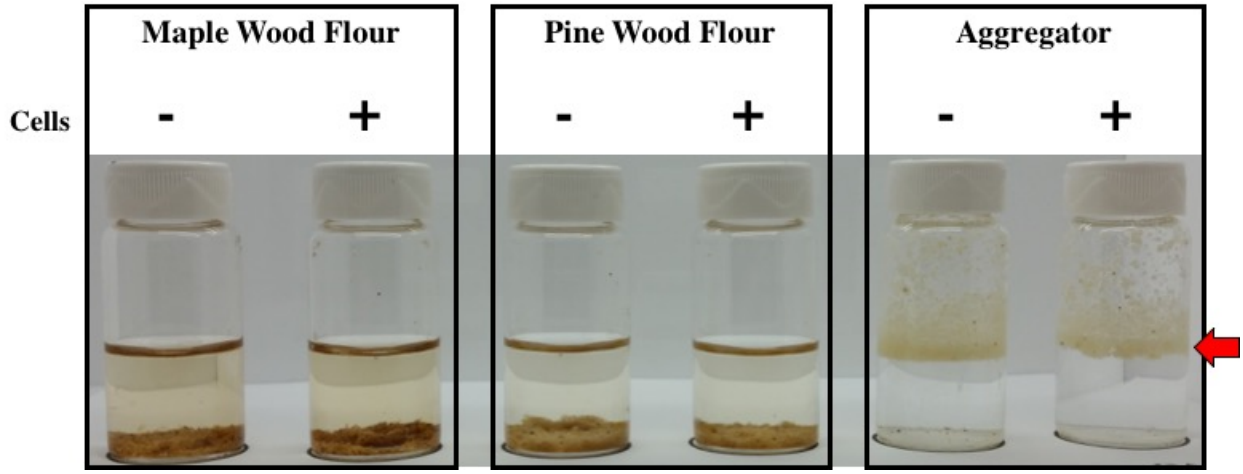

Figure 4.6. Buoyancy tests (Time 0); pictures were taken 30 minutes after mixing. Red arrow indicates the water surface level.

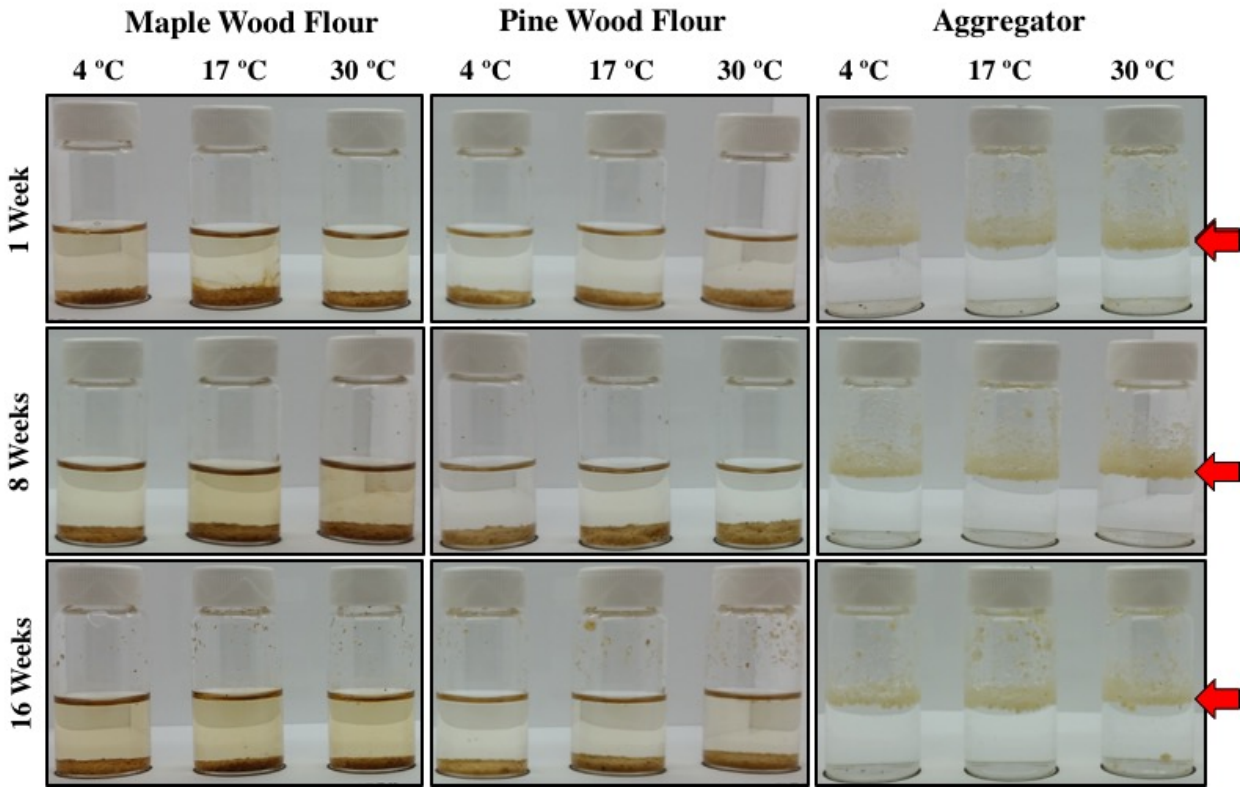

Figure 4.7. Buoyancy tests after 1, 8, and 16 weeks storage; pictures were taken 30 minutes after mixing. Red arrows indicate the water surface level.

4.1.2 Hydrocarbon Biodegradation Tests

A set of experiments was performed to assess whether the aggregator combined with oil-degrading bacteria and fungi would promote the biodegradation of crude oil in seawater. A second set of tests explored whether spiking the aggregator/microbial mix with a trace amount of oil prior to deployment ("priming") would lead a more rapid onset of biodegradation by reducing the lag phase of microbial growth: i.e., causing the cells to switch from metabolizing the simple polysaccharides found in the growth media used to produce the cells to the metabolism of oil hydrocarbons. Lag phase can last several minutes to many hours. In an open ocean setting, cells released into a marine environment might dissipate into the water column or die before adjusting to the presence of crude oil as a carbon/energy source for growth. Our hypothesis is that cells will remain on the surface of the aggregator and thus remain in contact with an oil slick and that priming could lead to a more rapid onset of biodegradation.

Mass spectrometry (described below) was used to monitor hydrocarbon biodegradation by microorganisms in the presence of the aggregator. Experiments were set up using a set of jars with an attached spigot at the bottom for easy sample collection with minimal disturbance of the seawater/aggregator interface: passing a pipet through the water surface to collect samples results in some oil sticking to the outside of the pipet and removal of an indeterminate amount of oil. Each jar contained 3 L of 0.22 μm filtered seawater and 30 μL of crude oil (West Texas Intermediate). A mixture of 3 microorganisms, *A. borkumensis*, *P. putida* and *H. resinae* (cell/spore suspension mixed at an approximately 1:1:1 cell density ratio), was prepared and washed with phosphate buffered saline (PBS) to prevent carryover of growth media. Each inoculum included either a priming (oil) or control (PBS) treatment 1 hour prior to inoculating the jars. The jars were inoculated with 2 g of aggregator mixed with 1.5 mL of the microorganism mix (with or without priming), Table 3.

Table 4.1. Composition of samples used to inoculate the oil biodegradation experiments. A volume of crude oil (priming) or PBS (control, no priming) was added to the aggregator 1 hour prior to inoculation.

Jar #	Material	Priming	Primer or Control Addition
1	Aggregator	No	0.1 mL 1x PBS
2	Aggregator	No	0.1 mL 1x PBS
3	Aggregator	Yes	0.01 mL crude oil
4	Aggregator	Yes	0.1 mL crude oil

For even mixing, each jar contained a magnetic stir bar and was placed on a stir plate during the 28-day oil biodegradation experiment. The jar lid was placed on top of the jar, not tightened down, to avoid creating anaerobic conditions inside the test jar. All jars were also wrapped in aluminum foil to prevent photo-degradation of hydrocarbon compounds.

Water samples were collected every 7 days during the 28-day experiment, and at each time point, the stir plates were turned off for10 minutes prior to sample collection. A 200 mL water sample was collected and spigots were rinsed with dichloromethane after sample collection to avoid potential hydrocarbon carry-over. Water samples were then spiked with surrogate recovery standards (5α-androstane, naphthalene-d8, acenaphthene-d10, phenanthrene-d10, chrysene-d12, and perylene-d12) followed by liquid-liquid extraction with dichloromethane.

The extracts were dewatered using sodium sulfate and carefully concentrated under a gentle N_2 stream to ~0.8 mL. Gas chromatography internal standards (d10-fluorene and d12-benzo[*a*]pyrene) were added to

the final concentrated extracts prior to gas chromatography-mass spectrometry (GC-MS) analysis. The GC-MS system is an Agilent 7890B GC interfaced to an Agilent 5977A MSD using electron impact in selective ion monitoring mode. A HP5-MS UI capillary column (J&W Inc., 30 m length, 0.25 mm diameter, 0.25 μm thickness) was used to achieve chromatographic separation of the alkanes and polycyclic aromatic hydrocarbons (PAHs).

Identification of target analytes was based on their retention times and confirmed by the abundance of a secondary ion relative to the molecular ion. PAHs included 16 unalkylated PAHs (the 16 EPA PAHs: naphthalene, phenanthrene, anthracene, fluorene, dibenzothiophene, fluoranthene, pyrene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[e]pyrene, benzo[a]pyrene, perylene, indeno[1,2,3-cd]pyrene, dibenz[a,h]anthracene, benzo[g,h,i]perylene), as well as 22 alkylated homologues of naphthalene, fluorene, dibenzothiophene, phenanthrene, anthracene, fluoranthene, pyrene, and chrysene.

Concentrations of dissolved unalkylated and alkylated PAHs in crude oil-spiked seawater were in the range of 0.02–3.00 ug/L (Figure 4.15), comparable to reported values in the literature (e.g., Prince et al. 2013). The concentration of the summation of 16 U.S. Environmental Protection Agency priority PAHs (16 PAHs) in time zero was 2.60 ug/L. Naphthalene and alkylated naphthalene had the highest concentrations. In general, the relative percentage difference (RPD) of PAHs concentrations from duplicate experiments were less than 20%, demonstrating that experimental data were reproducible.

As shown in Figures 4.15 and 4.16, PAHs were rapidly degraded. Most PAHs lost more than 50% of their concentration in the first 7 days; after that their concentrations were not noticeably changed. Fast biodegradation of crude oil in seawater was observed in different studies and more than 50% of total PAHs were biodegraded within 15 days (Prince et al. 2013; McFarlin et al. 2014; Venosa and Holder 2007). The biodegradation rate of crude oil in the presence of aggregator was compatible with other oil biodegradation studies (Prince et al. 2013; McFarlin et al. 2014; Venosa and Holder 2007). The published research supports our results and suggests that the aggregator would not interfere with biodegradation by microorganisms infused into the material and therefore it has a potential for use in oil bioremediation.

An identical set of experiments was also set up using pine wood flour instead of the aggregator and another set of experiments contained no aggregator or wood flour (the cells and any priming were added directly to the seawater), but these samples showed a very strong PAH background that obscured measurement of oil biodegradation (data not shown). It appeared that the crude oil stuck to the glass instead of being dispersed in seawater. The oil stuck to glass appeared to resist or limit biodegradation and was gradually released back into the water over time, overwhelming any signature of PAH degradation. The consistent PAHs degradation in the presence of aggregator might be because aggregator sorbed oil and ultimately prevented non-degraded oil from sticking to glass or dispersing into the water column, in effect, filtering the non-degraded oil from the water samples.

In addition to the assessment of oil biodegradation in the presence of aggregator, the effect of priming on biodegradation of PAHs was evaluated. Either 10 or 100 μL of crude oil (West Texas Intermediate) was added to aggregator and microorganism mix to enhance biodegradation by microorganisms. The entire mixture was incubated at room temperature for 1 hour prior to being placed inside the jar containing crude oil and seawater. As shown in figure 4.16, however, primed aggregator had more PAHs remaining and thus did not have the enhanced degradation expected. Instead, concentrations of some PAHs were increased over time and this might be because the crude oil used for priming was gradually released from aggregator mixture. Further optimization of priming microorganisms with crude oil is needed.

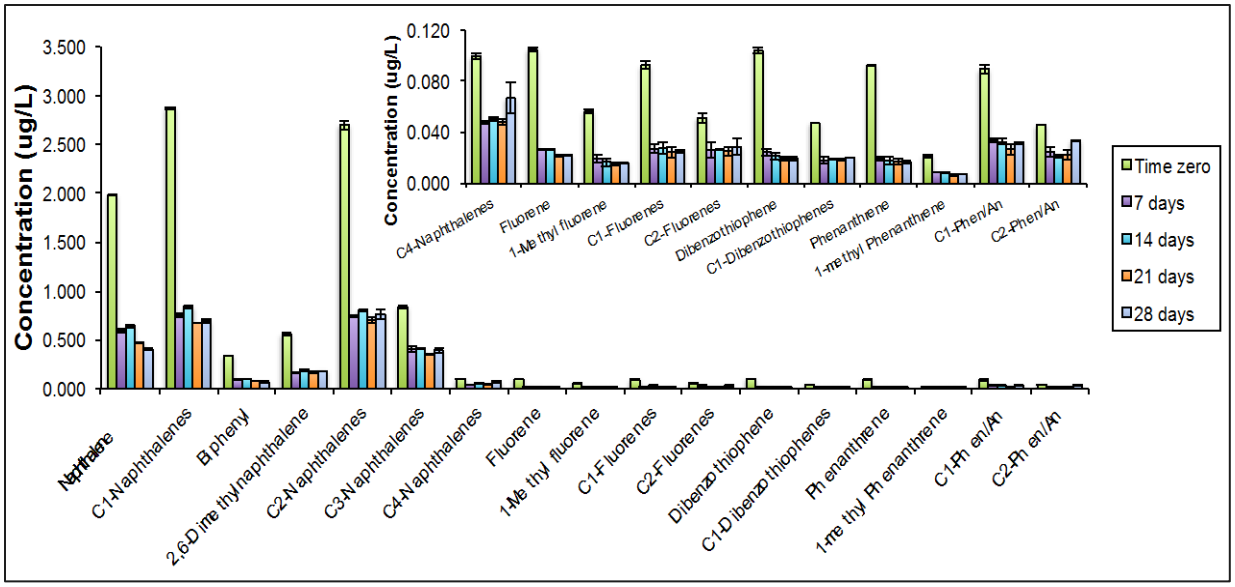

Figure 4.8. Concentration of PAHs in Crude Oil-Spiked Seawater, jars 1 and 2. PAHs concentrations were analyzed with samples that were exposed to aggregator and microorganism mix in crude oil-spiked seawater.

To determine if the remediation organisms, *A. borkumensis*, *P. putida*, and *H. resinae*, could survive or even grow in seawater without crude oil as a nutrient source, control experiments were performed to assess microbial viability in seawater without oil. Because *H. resinae* is a hyphal fungus (grows with multicellular threadlike structures) it was challenging to perform colony forming unit counts, so only *A. borkumensis* and *P. putida* were used for experiments.

Figure 4.17 shows an overview of the cell viability test experiment. Bacterial cells were grown until they reached the early stationary phase (*A. borkumensis* for 24 hours and *P. putida* for 8 hours), then they were washed in 4^{o}C sterile 1X PBS to prevent carryover of growth medium and resuspended in 4^{o}C sterile 1X PBS. Cell suspension was used to inoculate three different media types (Figure 4.18): sterile 1X PBS, rich medium (marine broth for *A. borkumensis* and nutrient broth for *P. putida*), and sterile filtered seawater. Media and bacterial cell mixtures were then incubated with shaking at room temperature for 7 days and samples were collected at time 0, 1 day, 4 days, 6 days, and 7 days. Collected samples were used for serial dilution and spread plates (Figure 4.19).

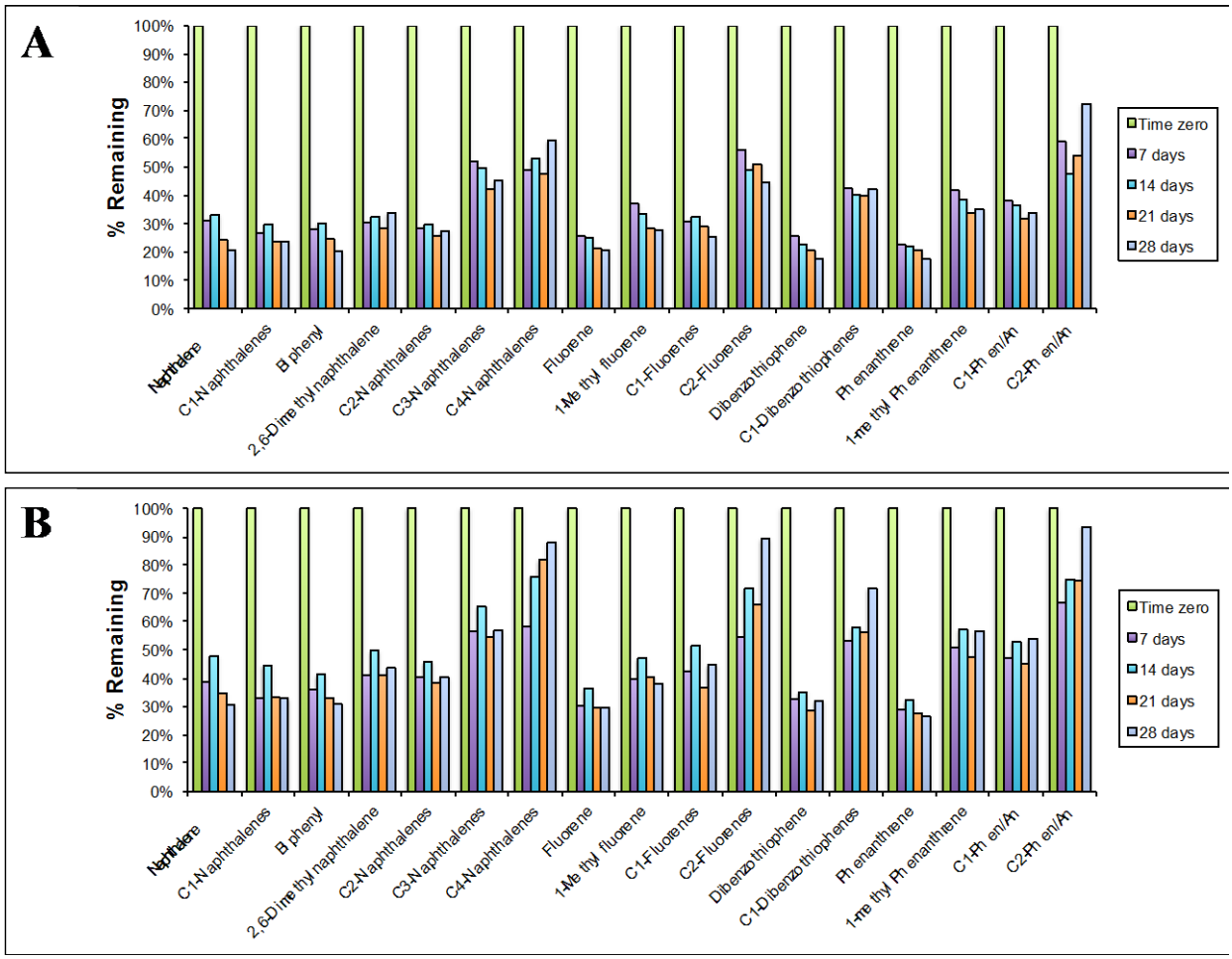

Figure 4.16. Percentage of PAHs Remaining in Crude Oil-Spiked Seawater. PAHs concentrations were analyzed with samples that were exposed to aggregator and microorganism mix in crude oil-spiked seawater: (A) no priming, (B) priming with 0.01 mL of crude oil (jar 3).

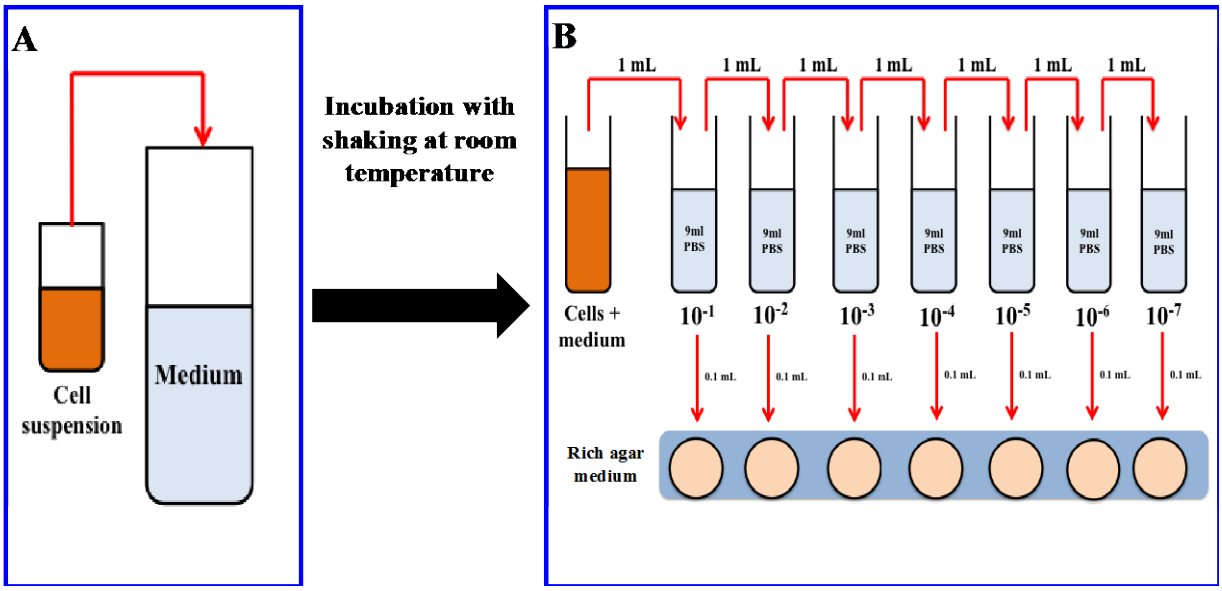

Figure 4.17. Cell Viability Experiment (Overview). To assess cell viability in different media, cells were grown until they reached the early stationary phase, then they were harvested to make a cell suspension. (A) Cell suspension was used to inoculate different media. Mixtures were incubated with shaking at room temperature. (B) Samples were collected at five different time points (Time 0, 1 day, 4, 6, and 7 days) and used for serial dilutions and spread plates.

Figure 4.18 shows the colony forming unit counts results. *A. borkumensis* and *P. putida* showed different viability trends and that might be because of differences in their metabolisms.

In sterile seawater, both *A. borkumensis* and *P. putida* started to lose viable cells after 1 day. It appeared that *P. putida* survived better than *A. borkumensis* (Figure 4.18C and F).

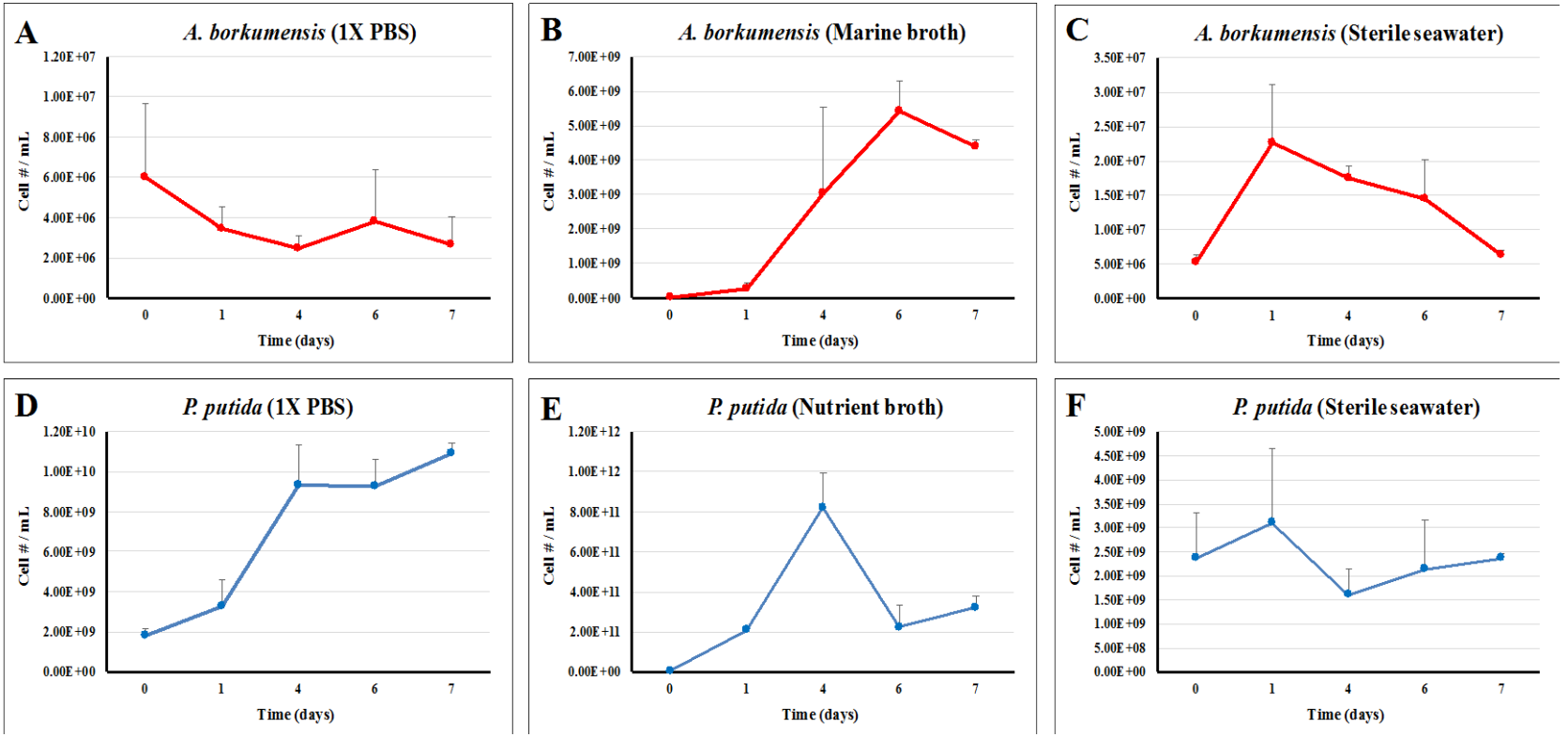

Figure 4.18. Cell Viability Tests (*A. borkumensis* and *P. putida*). *A. borkumensis* (A~C) or *P. putida* (D~F) cells were used to inoculate three different media: sterile 1X PBS, marine broth, and sterile seawater. Mixtures were incubated with shaking at room temperature and samples were taken at five different time points (time 0, 1 day, 4, 6, and 7 days). Collected samples were used for serial dilutions and spread plates to calculate concentrations (# of cells/mL of media) at each time point. All experiments were done in triplicate and average values are shown. Standard deviation values are represented by error bars.

To briefly check cell viability after the 28-day oil biodegradation experiment, seawater samples were collected from 12 different jars (Table 4.1) to spread over three different agar media types. For the oil biodegradation experiment, all seawater samples were spiked with crude oil (West Texas Intermediate) and microorganism mix (*A. borkumensis*, *P. putida* and *H. resinae*).

A 0.1 mL seawater sample was used on the agar plate spread. Plates were incubated at room temperature and the growth on plates was compared to that on pure culture plates. All plates showed visible growth and all three representative microorganisms appeared to be present based on visual identification.

5.0 Herding and Sorbent Studies in Icy Water

Ice formation can begin in different ways. Within non-turbulent environments, a surface ice cover forms in a fairly simple manner that may not involve more complex ice formations. However the formation of ice in turbulent waters (caused by wave action of flowing over and around structures or the bottom of relatively high gradient rivers and streams) originates with turbulence and frazil ice. Brown et al. explain that, in nature, supercooling of the water "occurs when little or no surface ice is present, the air temperature is sub-freezing, and the water flow [or wave action] is sufficiently turbulent to overcome stratification" (Brown et al., 2011). Turbulence causes tiny ice crystals formed at the surface to become suspended in the water column and these crystals act as nucleation sites for further ice crystal growth. This suspension of small, actively growing ice crystals, or frazil ice, can be particularly problematic as it can adhere and grow on other surfaces such as oil skimmers and booms. Of particular concern to the development and testing of the aggregator was the potential for ice to grow on the surface of the aggregator and thus impede its interaction with surrounding oil.

Bureau of Safety and Environmental Enforcement aggregator experiments were conducted in a dedicated walk-in freezer laboratory. The walk-in Arctic simulation laboratory (shown below) operates at air temperatures down to -15°C. Additionally, a recirculating chiller that cools polyethylene glycol to -30°C is incorporated with the freezer to provide rapid, supplemental chilling to the seawater tanks. Aggregator tests under simulated Arctic conditions were conducted in a Pyrex circular glass dishes (diameter: 150 mm, height: 75 mm) and in a 1100 L oval raceway that was filled with unfiltered seawater, to which crude oil and aggregator were added. A paddle wheel and recirculating pumps provided turbulence in the raceway to facilitate the formation of frazil ice. All ice used in this study was created by chilling unfiltered seawater to below freezing temperatures in the test dish or tank. Figure 5.1 shows an overview of the Arctic test capability at MSL; Figure 5.2 shows a close-up of the 1100 L raceway used in this study.

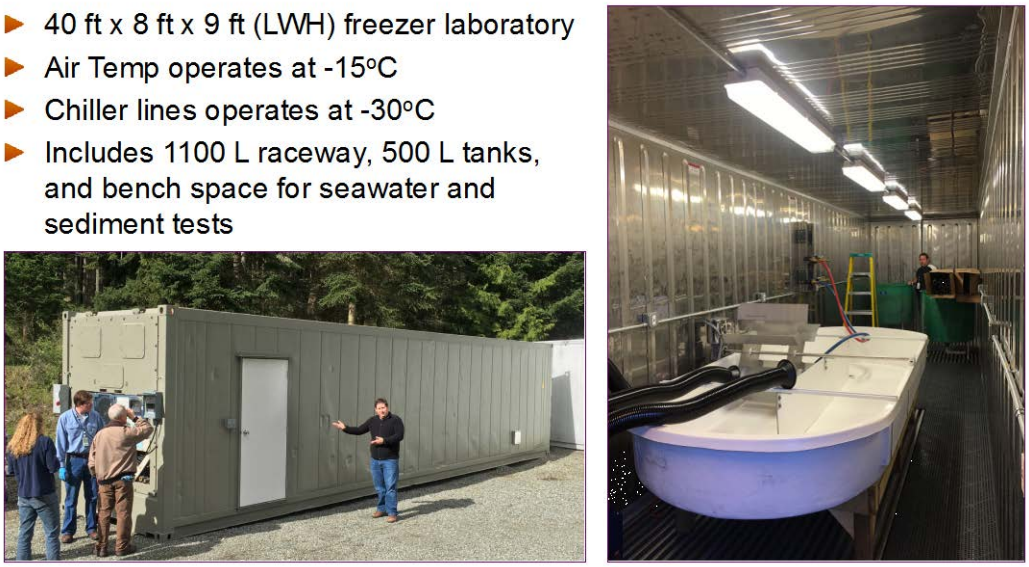

Figure 5.1. Freezer Laboratory. The freezer laboratory at MSL operates at air temperatures down to -15°C. A closed looped chiller line operates -30°C. The laboratory is highly flexible, can be fitted with a variety of tank types and sizes, and is permitted to operate with crude oil and dispersants.

The 40-mesh pine OA aggregator was tested for buoyancy, oil sorption, and water repellency under freezing conditions and in the presence of sea ice. A total of 500 mL of crude oil was dispensed onto a ¼ to ¾-inch surface of frazil ice in the 1100 L tank (Figure 5.3). As the oil was dispensed, a portion of the oil went under the ice, some stayed at the seawater surface (some ice was displaced by the oil), and some oil ended up directly on top of the ice. The aggregator was dispensed on top of the oil/ice/seawater interface. The aggregator, with or without sorbed oil, remained buoyant on top of the ice and seawater. A large portion of the oil was immediately sorbed by the aggregator; after 15 minutes and further examination of the oil spill, it was observed that the aggregator had pulled the oil from the surface of the ice (Figure 5.4 and Figure 5.5). Areas of the ice covered with oil at time 0 became clear as the oil migrated to the aggregator. As unbound oil reached the surface of the turbulent ice and seawater, it was also bound to the aggregator and thus captured at the surface. There were no discernable signs that the aggregator was affected by the simulated Arctic conditions.

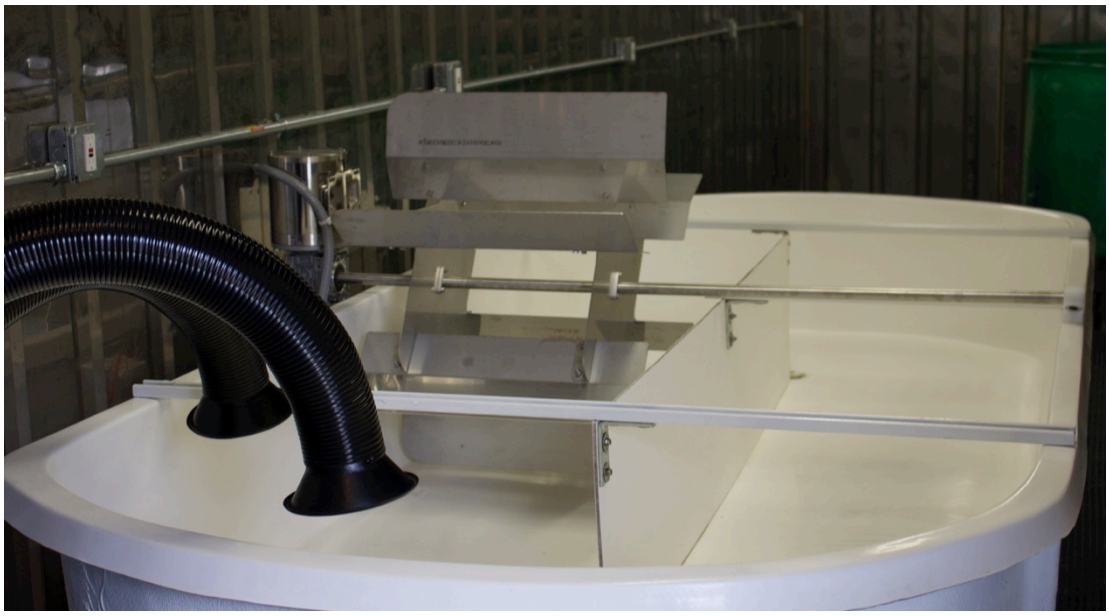

Figure 5.2. 1100 L Test Tank in the Arctic Lab. The 1100 L tank was used for testing aggregator with frazil ice. The paddle wheel at the far left operates with clockwise and counterclockwise rotation. Tank dimensions are 138” (L) x 60” (W) x 16” (H). The black hoses to the bottom left of the picture are part of an air filtration unit.

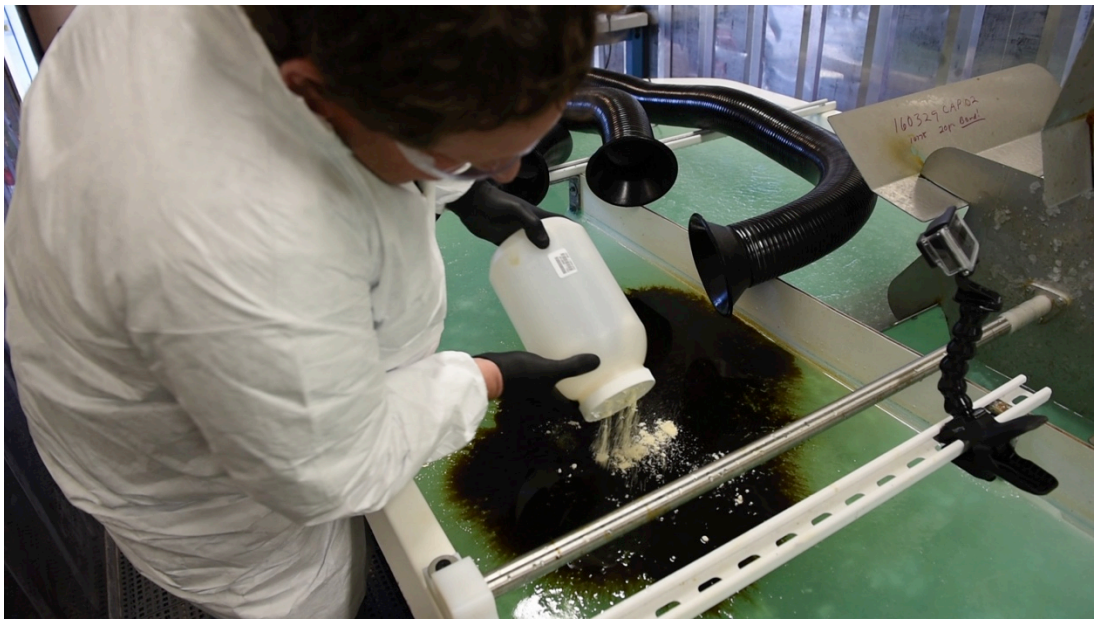

Figure 5.3. 1100 L Test Tank in the Arctic Lab: Frazil Ice + Oil + Aggregator. Aggregator added to 500 mL of crude oil that was dispensed into frazil ice. The aggregator was unaffected by the presence of the ice. It was able to sorb oil from the surface of the water, on top and below ice. No ice formation was seen on the aggregator.

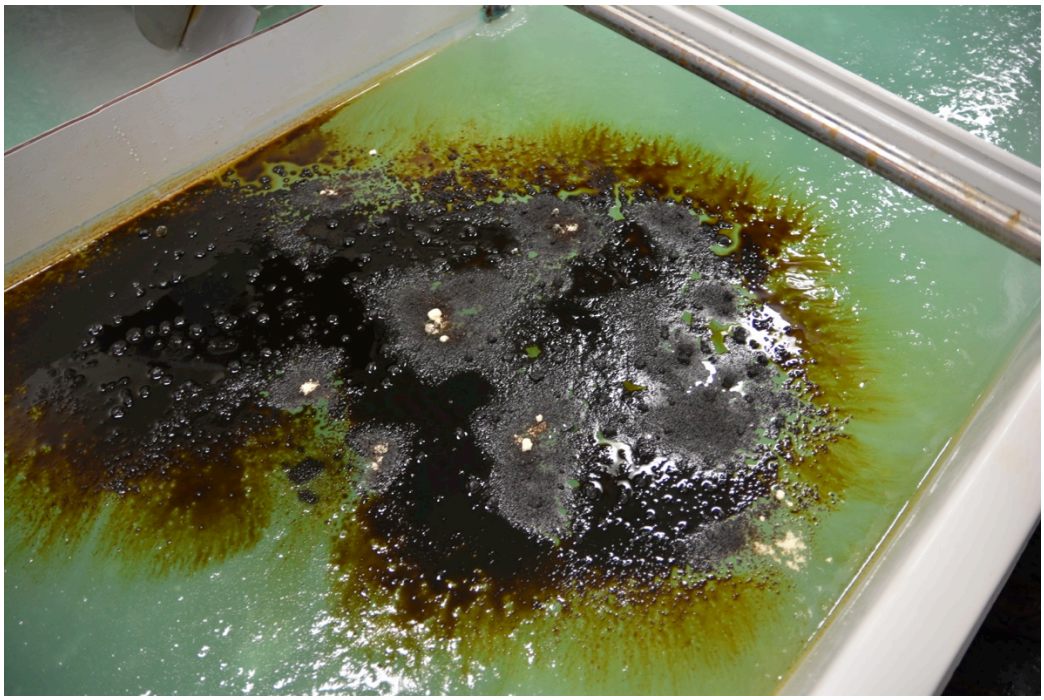

Figure 5.4. Oil + Aggregator + Ice Close-Up. The aggregator is unaffected by the ice. Areas of the ice that were covered with oil before the aggregator was added clear up as the aggregator pulls oil off the ice toward the aggregator.

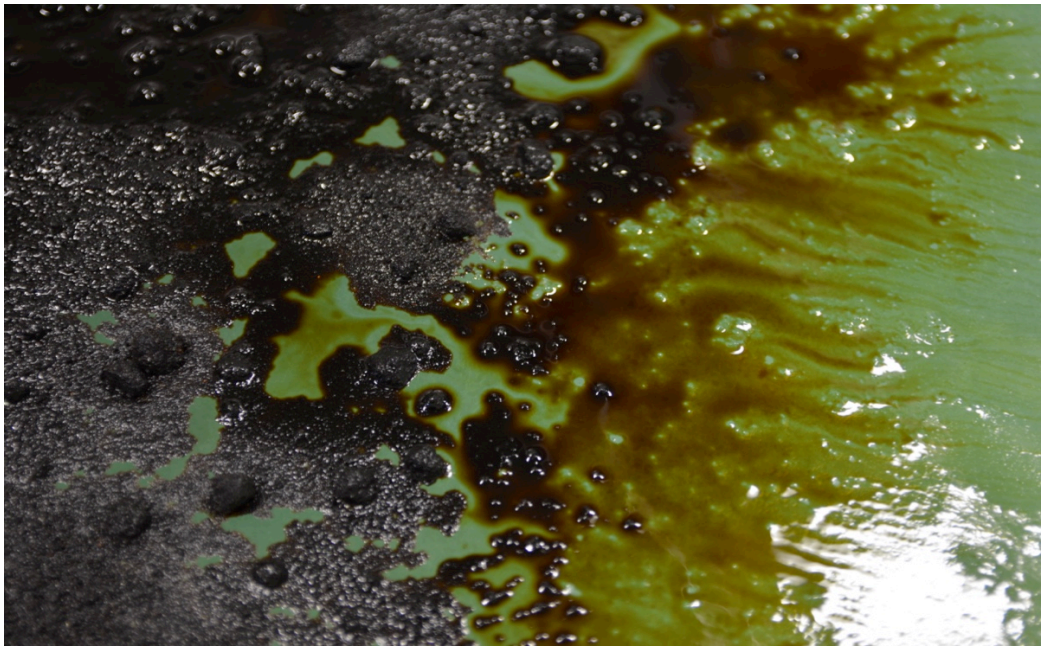

Figure 5.5. Oil + Aggregator + Ice Close-Up 2. A close-up of the aggregator dispensed on top of the crude oil and frazil ice. The aggregator removed oil from the surface of seawater; as this happened, the seawater became visible between the dispensed aggregator.

A 24-hour experiment was conducted to determine whether ice would crystalize on the aggregator's surface when exposed to sub-zero conditions over an extended time period. This experiment tested the

aggregator in the presence of frazil ice and ANS or Dorado crude (Figure 5.6 and Figure 5.7). Crude oil was dispensed onto the frazil ice surface and then the aggregator was added to achieve an oil:aggregator ratio of 10:1. After 24 hours, no ice was observed forming on the surface of the aggregator (Figures 5.8 and 5.9). Figures 5.8 and 5.9 show a side view image of the aggregator/oil mixture on top of the ice layer. This is not unexpected, because the extreme hydrophobic chemical properties of the aggregator repel water from its surface, inhibiting ice formation by not allowing a nucleation site.

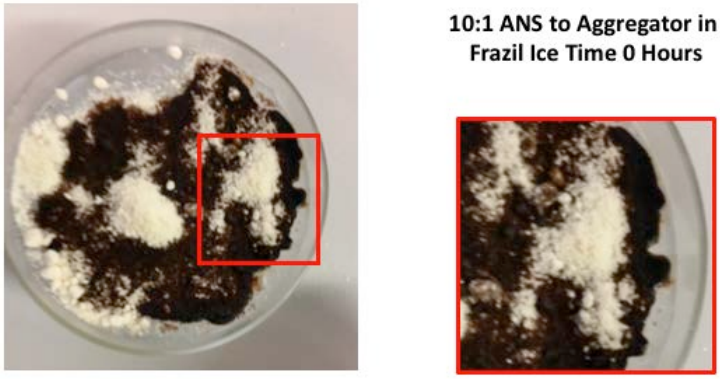

Figure 5.6. Frazil Ice/Aggregator ANS Oil at Time 0. ANS crude oil and aggregator was added to Frazil ice to determine whether ice would nucleate on the aggregator over a 24-hour time period. Aggregator sorbed oil from the ice when dispensed. Cold temperature and ice had no

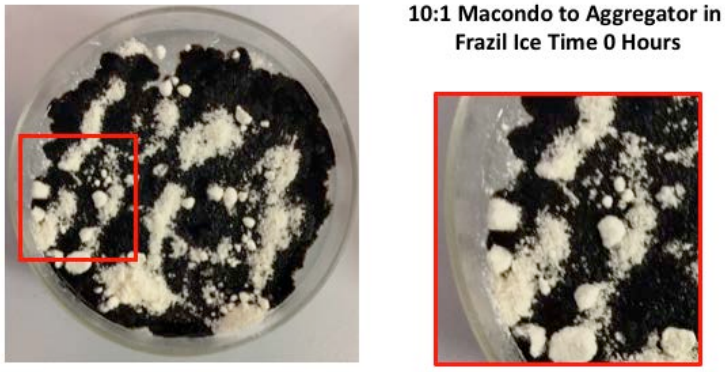

Figure 5.7. Frazil Ice/Aggregator Dorado Oil at Time 0. Dorado crude oil and aggregator were added to frazil ice to determine whether ice would nucleate on the aggregator over a 24-hour time period. Aggregator sorbed oil from the ice when dispensed. Cold temperature and ice had no observable effects on the aggregator compared to the warm water test.

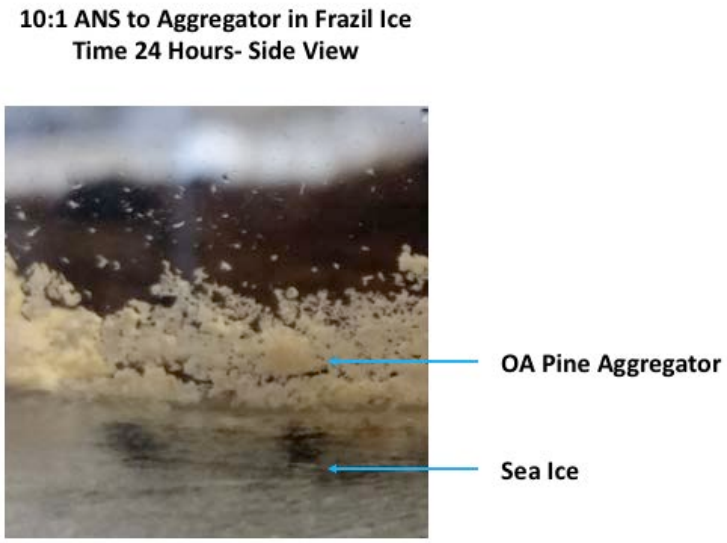

Figure 5.8. Frazil Ice/Aggregator ANS Oil at Time 24. A side view that looks through the glass test vessel allows for close-up observation of the ice/oil/aggregator interface. Sea ice has built up under the oil and aggregator mixture. This picture taken after 24 hours of exposure shows no ice formation on the aggregator.

Figure 5.9. Frazil Ice/Aggregator Dorado Oil at Time 24. A side view that looks through the glass test vessel allows for a close-up observation of the ice/oil/aggregator interface. Sea ice has built up under the oil and aggregator mixture. This picture was taken after 24 hours of exposure shows no ice formation on the aggregator.

Additionally, aggregator performance was not observably affected by either the sub-zero air temperature or the presence of frazil ice in seawater. Buoyancy, oleophilicity, and hydrophobicity properties remained unchanged, even though the viscosity of the oil increased with the lowering of temperature. The oil and aggregator formed a heterogeneous clump at the surface that remained throughout the experiment.

6.0 Conclusions and Recommendations

The objective of this project was to develop an alternative product for use in oil spill response mitigation. Traditionally, chemical herders have been used to make crude oil slicks contract into a slick of sufficient thickness to facilitate in situ burns. Unfortunately, chemical herders are affected by moderately rough seas and wind speeds greater than 5 mph, dissipate into the surrounding seawater, and many are toxic to some aquatic flora and fauna. PNNL developed a product "aggregator" that solves many of these problems.

6.1 Conclusion

Preliminary results indicate that the PNNL-developed aggregator is unaffected by adverse environmental conditions: turbulence from wind or waves has little effect on the product and it stays buoyant for months. Furthermore, the product is a non-toxic, environmentally friendly product that can actually support bio-remediation of crude oil with the addition of microorganisms that eat oil.

After testing a number of wood species and mesh sizes, 40-mesh pine wood flour was selected as the base species for the aggregator. The wood flour was grafted on the surface with fatty acid (OA) by an esterification reaction to develop an innovative aggregator. The OA-modified aggregator is both hydrophobic and oleophilic—two chemical properties that enable the aggregator to adsorb and retain crude oil very well and to remain buoyant in seawater for extended periods (months). Other modifications of wood flour, such as by thermal treatment or acetylation were found to be less effective at producing the desired traits of oil sorption, water repulsion, and buoyancy.

Lab and mesoscale testing showed that the aggregator had a strong attraction to crude oil and the ability to withdraw oil directly from seawater. The aggregator has a strong attraction to oil until it reaches saturation, approximately five times its mass in oil with the formulation presented in this study. The strong attraction for oil was apparent when a small quantity of aggregator that had inadvertently escaped into the large burn tank at JMTF removed the oily sheen from the seawater surface by the following day. We hypothesize that the oleophilic and hydrophobic properties help to facilitate ISB of crude oil that is less than 1 mm thick. At an oil thickness of less than 1 mm and in the absence of aggregator, the spilled oil cannot produce enough heat to maintain combustion because the underlying seawater acts as a heat sink and some water becomes mixed with the oil. The aggregator, however, appears to wick the oil from the surface, creating enough of a barrier from the seawater to support combustion of thin layers of oil. In icy water, the aggregator was effective at separating oil from seawater and ice and at facilitating burn ignition and increasing the intensity of the burn.

While the newly developed aggregator supported burning small, thin, and discontiguous (i.e., due to the presence of ice) spills, its ability to act as a chemical herder facilitating the contraction of a slick was minimal. In laboratory-scale tests, the aggregator did pull oil together from the surface of the water into a single, cohesive amalgamation, but this effect was only observed when small quantities of oil were tested. As the volume of oil and surface area of the slick was increased, this effect was diminished. The physical size of the solid aggregator particles may limit the extent to which the combined oil and aggregator slick is able to contract. One interesting finding was that when applied to the perimeter of a slick, the aggregator appeared to create a barrier that limited the further expansion of the slick. The strength and resilience of this effect relative to the viscosity and spreading force of the oil was not tested.

Further research and development are needed to improve the formulation and synthesis of the aggregator, to further characterize its performance, and to explore different methods of use and deployment. Overall, the new aggregator proved successful as an alternative approach to facilitating ISB under challenging conditions: rather than forcing oil to coalesce into a thicker slick to enable burning, the aggregator enables burning of thinner slicks, particularly in cold or icy water. Furthermore, burning may ensue within minutes of application, facilitating a rapid response and reducing the risk that wind, waves, or ice might disrupt a burn. When aggregator is used, the resulting burn residues appear to be more buoyant, which should allow for better biodegradation or collection by booms or skimmers. Additionally, the aggregator is an effective means for stabilizing and delivering bioremediation microorganisms. In conclusion, the aggregator may provide a more resilient (to challenging marine conditions), adaptable, non-toxic and ecologically friendly alternative to conventional herders.

6.2 Aggregator Performance Overview and TRL Tables

Table 6.1 provides an overview of aggregator performance and the Technology Readiness Level (TRL) at the completion of project E15PG00037. TRLs were determined using the Bureau of Safety and Environmental Enforcement oil spill TRL table.

Table 6.1. Aggregator Performance and TRL Level

Aggregator Performance	Low	Medium	High
Oil Sorption			✖
Herding of Oil	✖		
Bio Remediation Platform			✖
Supports Burn Less <1mm		✖	
Works In Ice and Sub Zero Temperatures			✖

TRL Level of Progression for BSEE Aggregator Project E15PG00037	
Start of Project	TRL 1
End of Project	TRL 3

6.3 Recommendations

This section identifies a number of questions identified during the course of study and possible next steps.

- While modification of sawdust with OA has shown promise, X-ray diffraction and thermogravimetric analysis suggest that improvements can be made to the aggregator to boost performance. Improvements may also be possible to decrease the cost of synthesis, including means of recycling some of the reagents.
- Explore whether adding a dry accelerant to the aggregator increases burn temperatures, improves burn efficiency, reduces ignition temperatures, or assists with burning weathered oil. Preliminary testing with Mg added to the aggregator mix resulted in an increase in temperature, albeit small. Further optimization could result in a significant increase in burn temperatures.
- Examine how the aggregator may be used to create a barrier to limit the spread of a surface oil slick (i.e, a sprayable boom)
- Examine alternative means of application for both large and small spills. Is it possible to incorporate the aggregator into or with another material such that it would not be released as a loose powder?

- Examine how long and how completely the aggregator retains sorbed oil in an open water setting when partially or fully saturated.
- Determine how compatible oil soaked aggregator is with skimming operations. Does the thickening of the slick and oil-water separation aid in the efficient removal of oil from the water surface? Can oil be separated back off of the aggregator?
- Further exploration of burning and bioremediation in icy waters. Does the aggregator facilitate bioremediation of oil by keeping it at the water's surface where it may be warmer (through solar heating) and more oxygenated? Conduct a more intensive examination of burn efficiency; explore the use of larger scale burn studies at MSL, Poker Flats, or another location.
- Long-term fate of unbound aggregator in the marine environment: how quickly does it degrade. Also examine the composition and fate of burn residues when aggregator is used.
- Use of the aggregator in other applications: treatment of terrestrial spills, including on ice, snow, or permafrost in the Arctic. Use in industrial settings.

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Appendix A

Glossary

Appendix A

Glossary

cDNA

Complimentary DNA is the synthesis of nucleic acid sequences from an RNA template. The synthesis is a technique used in molecular biology studies.

esterification reaction

The chemical process of making ester compounds and chemical reactions. An acid is commonly used to speed up the reaction time in the chemical process.

oleic acid

An odorless fatty acid with an 18-chain carbon structure. Oleic acid is found in animal and vegetable fats.

oleophilicity

A material or substance that has an affinity for oil.

PCR (polymerase chain reaction)

PCR is a technique used to replicate a segment of DNA into millions of copies. The process tan be used to target a specific sequence for replication, or may be modified to allow for little or no specificity.

primer

A short sequence of single-stranded DNA that is complimentary to a sequence of DNA and subsequently binds to that sequence. The primer is then used as a starting site for gene or sequence duplication by PCR.

species-specific primers

A pair of primers that are designed to bind to a pair of complimentary DNA sequences that is unique (either each primer individually or as a pair) to a given species. PCR with these primers will only amplify the target sequence from the target species. These primers are often used to quantify a single organism or gene from an environmental or mixed community sample of DNA.

wood flour

Refined sawdust that is milled to a specific mesh size. Commonly used in the production of composite wood products.

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