

The effects of micro-algae characteristics on the bioremediation rate of Deepwater Horizon crude oil

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Summary

Crude oil spills have extensively harmed ecosystems. Current spill treatments are limited by high costs, hazardous by-products, and unsustainability. Algae crude oil bioremediation has been an obscure field in research. We sought to examine the potential of algae bioremediation as a treatment for crude oil spills. This study investigated whether different algal characteristics would influence the degradation rates of Deepwater Horizon crude oil. Eight algae were utilized: *Aphanocapsa* sp., *Chlorella autotrophica*, *Coccochloris elabens*, *Dunaliella tertiolecta*, *Oscillatoria* sp., *Scenedesmus obliquus*, *Synechococcus elongatus*, and *Volvox aureus*. Algal growth in heavy crude conditions was analyzed via spectrophotometry and computer image processing techniques. We hypothesized that motility, levels of chlorophyll a & b, and multicellularity would affect algae bioremediation rates. Test tubes containing algae and control test tubes that lacked algae, placed under fluorescent lighting, contained freshwater or seawater solution and the crude oil. Crude oil and water levels were recorded for fifteen days. The data was analyzed for correlations between the specific algal characteristics and degradation rates. Results revealed the algae degraded up to a significant 35% of the crude. Correlations between algal characteristics and bioremediation rates were not observed, so the hypotheses were unsupported. However, two important conclusions were deduced: algae can degrade "heavy" crude oil and they can thrive in high crude conditions, abilities that are necessary for bioremediation. From this study, we have demonstrated that properly-controlled algae are viable agents for crude oil bioremediation.

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Introduction

The effects of globally-occurring crude oil spills are devastating. The current methods of oil spill cleanup are helpful but present drawbacks (1, 2, 3). An alternative method of treatment must be utilized in conjunction with current methods to ensure success and

efficiency. A potential and innovative treatment is algae bioremediation; however, the details concerning algae bioremediation are still vague. We sought to expand the knowledge regarding algae bioremediation by testing the effects of algal characteristics on algae's bioremediation rate of Deepwater Horizon crude oil.

Bioremediation is the employment of natural processes to remove harmful chemicals and contamination in the environment. Algae bioremediation is unique because it is a self-sustaining cycle (Figure 1). To oxidize contaminants into less-harmful metabolites, algae extract and utilize oxygen from its surrounding environment; these metabolites include CO_2 and H_2O . For growth, algae use photosynthesis, which requires CO_2 and H_2O . Photosynthesis, in turn, releases oxygen that algae can employ for further contaminant oxidation, thus repeating the cycle.

Oil spills devastate ecosystems and the atmosphere by tainting water sources, releasing toxic vapors, and causing irreversible contamination to aquatic and terrestrial habitats (2). For ecosystems, crude oil contamination directly kills producers and consumers within the proximity of the spill (4). Consumers not directly affected are detrimentally impacted by the spill via depletion of their crucial food, energy, and water sources; this depletion results in a domino effect of mortality throughout the food web and transforms healthy ecosystems into dead zones (2). Moreover, some hydrocarbons are semi-volatile; they evaporate into the air and create heavy vapors that reside near the ground, causing hazardous conditions that may subsist for decades. During the Deepwater Horizon spill, the Natural Resources Defense Council reported that

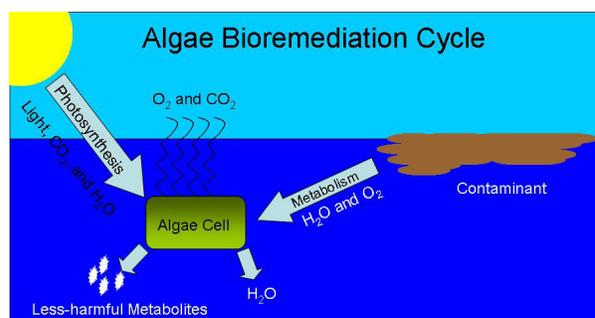


Figure 1. Algae Bioremediation. Metabolism of the contaminants through algae produces CO_2 , H_2O , and less-harmful components. In turn the algae are able to use the CO_2 , H_2O , and less-harmful components as nutrition necessary for photosynthesis and sustainability.

Test	Avg. rate of mass decrease (g/day)	Avg. rate of water decrease (g/day)	Avg. rate of degradation (g/day)	% crude oil degraded
Freshwater Control	0.13964	0.14286	-0.00322	~0%
Seawater Control	0.15036	0.15357	-0.00321	~0%
<i>Coccochloris elabens</i>	0.23000	0.20714	0.02286	34.99%
<i>Scenedesmus obliquus</i>	0.16571	0.14642	0.01929	29.53%
<i>Oscillatoria</i> sp.	0.25750	0.24287	0.01463	22.41%
<i>Dunaliella tertiolecta</i>	0.15678	0.14500	0.01178	18.05%
<i>Chlorella autotrophica</i>	0.20964	0.2000	0.00964	14.76%
<i>Aphanocapsa</i> sp.	0.24142	0.23571	0.00571	8.74%
<i>Synechococcus elongatus</i>	0.2150	0.20714	0.00536	8.20%
<i>Volvox aureus</i>			Inconclusive	

Table 1. Average degradation rates of eight algae and two controls

citizens of Louisiana suffered from nausea, vomiting, headaches, and labored breathing caused by heavy oil vapors (4). Other hydrocarbons are carcinogenic and irritating to the skin and airways; with prolonged contact, people can develop acute health problems (5, 6).

In addition, oil spill cleanup has not been completely successful in the past. For example, in 1989, the Exxon Valdez spilled 0.3 million barrels of crude oil off the coast of Alaska. Today, decades later, oil still exists and causes damage in subsurface reservoirs and on coastal beaches (7). While the media heavily scrutinizes oceanic crude oil spills, 28-30% of crude oil spills occur overland and affect freshwater sources (8). Consequently, the noted detrimental impacts of oil spills affect terrestrial and aquatic habitats and impact all areas of the globe.

Three prominent methods have been utilized for oil spill cleanup: mechanical pumping, combustion, and bacterial bioremediation. When the Deepwater Horizon spill escalated, the oil company BP used oil skimmers to remove and treat crude oil-contaminated water from the Gulf of Mexico. However, this method accumulates large energy and production expenditures. Later, the combustion of buoyant oil was used but this method produced dangerous fumes (1). Scientists have also searched for alternatives in bacterial bioremediation; some bacteria, such as *Pseudomonas* sp., are effective but many require long adaptation periods and dangerously decrease oxygen saturation levels when metabolizing hydrocarbons (2, 3). Because of these reasons, the use of bacteria is questionable for widespread remediation. While these previous methods and others have provided some benefit, they possess a variety of disadvantages.

Researchers have found that algae can oxidize many types of hydrocarbons into less harmful components, hinting at their potential to degrade heavy crude oil, which includes multiple hydrocarbons (3, 8, 9, 10, 11, 12, 13, 14). However, an analysis of algae is lacking. What algal characteristics make one alga more effective than another at crude oil degradation?

This project explored, as the next step in advancing

algae bioremediation, correlations between integral algal characteristics and the rate of hydrocarbon degradation. Diversity in algal characteristics plays significant roles in algae's ability to grow, adapt, and reproduce in different habitats (15). It may also influence the rate of algal crude oil bioremediation.

The purpose of this study was to evaluate the effects of three algal characteristics (motility, chlorophyll type, and cellular structure) on the algae bioremediation rate of Deepwater Horizon crude oil to determine whether these traits play significant roles in increasing the rate of algal crude oil degradation. Another aim was to study the growth of algae exposed to "heavy" (dense and solid) crude oil introduced in high proportions. This aim would determine whether algae are suited for the bioremediation of large-scale, highly viscous crude oil spills.

The central hypotheses are that differences in motility, levels of chlorophyll a & b, and multi-cellular formation would change the rate of algal crude oil degradation. Motile algae could move through the solution to collect and oxidize more crude oil as compared to non-motile algae. Next, having both chlorophyll a & b in contrast to only chlorophyll a could improve the rate of crude oil degradation because algal oxidizing agents depend on photosynthesis (16). Together, chlorophyll a & b capture a larger range of light waves than chlorophyll a alone, perhaps allowing for more oxidization from increased photosynthetic activity. Finally, multi-cellular algae have a higher surface area compared to unicellular algae. This higher surface area would conceivably allow multi-cellular algae to capture and degrade more crude oil in less time as compared to unicellular algae.

Results

We tested the abilities of two freshwater and six saltwater algal species to degrade crude oil. After adding crude oil to each algae sample and allowing a degradation period of 15 days, the degradation rates for each alga were determined through an equation described in the Materials and Methods section. Then

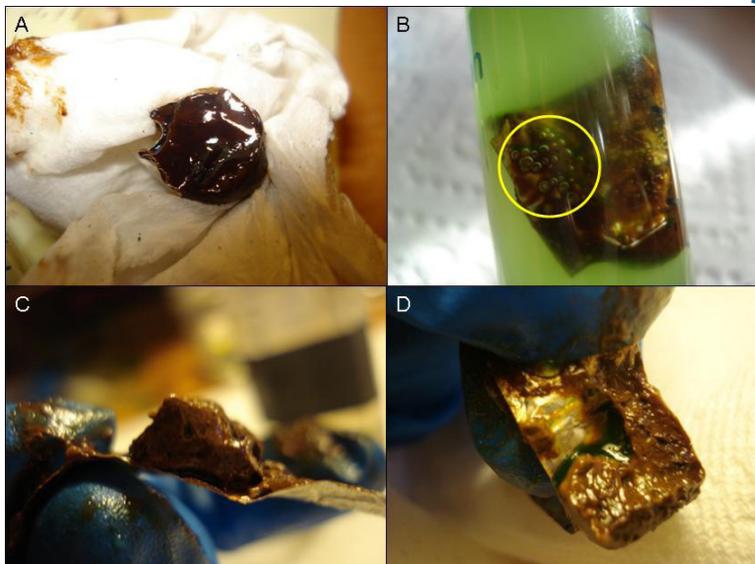


Figure 2. Algal Degradation. **A.** Seawater Control. This crude oil did not experience degradation after 15 days. It was still very dark, solid, and smooth. **B.** *Scenedesmus* Trial. The little white dots on the aluminum cover show areas of degradation. Gaseous metabolites from hydrocarbon oxidation are circled in yellow. **C.** *Coccochloris* Trial. The crude oil lost its circular form and dark color due to algal degradation. The algae formed deep pores within the crude oil. **D.** *Coccochloris elabens* Trial. *Coccochloris elabens* created crevices and pores, grew significantly inside, and degraded applied crude oil.

the rates were compared to the characteristics of the algae to look for a correlation between the rates and the characteristics. Furthermore, algae growth was analyzed via a spectrophotometer and by looking at algae concentration.

Only the gaseous metabolites could be accounted in Δm_{test} for through the proposed degradation equation. Because the project tested for mass, solid metabolites would still reside in the solutions and contribute to the mass. However, gaseous metabolites would leave the solutions.

The degradation results are shown in **Table 1**. Crude oil degradation by the algae was evident because there was a positive difference calculated by using the degradation equation. The control tests showed no degradation through the equation.

The top four bioremediation algae based on averages were *Coccochloris elabens*, *Scenedesmus obliquus*, *Oscillatoria* sp., and *Dunaliella tertiolecta*. *C. elabens* degraded the most crude oil in 15 days, an approximate 35% of the oil at an average rate of 0.02286 g per day. *S. obliquus*, *Oscillatoria* sp., and *D. tertiolecta* degraded 29.5%, 22.4%, and 18.0%, respectively. The remaining algae, except *Volvox aureus*, “moderately” degraded crude oil based on comparisons between the tests. *V. aureus* had trouble adapting to its solution and did not start growing until after the tests were finished, so its degradation rate of crude oil was inconclusive.

The algal degradation of the crude oil was confirmed through visual analysis (**Figure 2**); algae formed holes in the crude oil, permeated deeply into the substance, and

produced visible, solid hydrocarbon metabolites. The controls showed no such signs of bioremediation.

The graphs analyzed to determine correlations between a characteristic and the rate of algal crude oil degradation are shown in **Figure 3**. The top four performing algae had no single characteristic in common, so no correlations were found.

All algae showed exponential growth (**Figure 4**) except for *Dunaliella tertiolecta*, which exhibited a 28.3% biomass decrease. This decrease could have been due to a salinity insufficiency as *D. tertiolecta* needs 150 times average seawater salinity levels (~35 ppm) to thrive. As mentioned before, *V. aureus* showed signs of growth after its tests but seemed stagnant during the duration of the tested fifteen days; shown in spectrophotometry, *V. aureus* solutions had 24% absorbance compared to the water control absorbance of 0%. Extrapolation from this data yielded that *V. aureus* needed a longer adaptation period than the other tested algae to grow in crude oil environments. *Oscillatoria* sp., because it was clumped together and unevenly spread out within its medium, was incapable of being analyzed for growth via spectrophotometry and computer image processing (CIP) techniques. But, through visual observation of color and algae density, it was evident that *Oscillatoria* sp. had significant increases in growth.

Discussion

This study showed that algae can significantly degrade crude oil (up to 35% in 15 days), but it did not reveal correlations between algal characteristics and

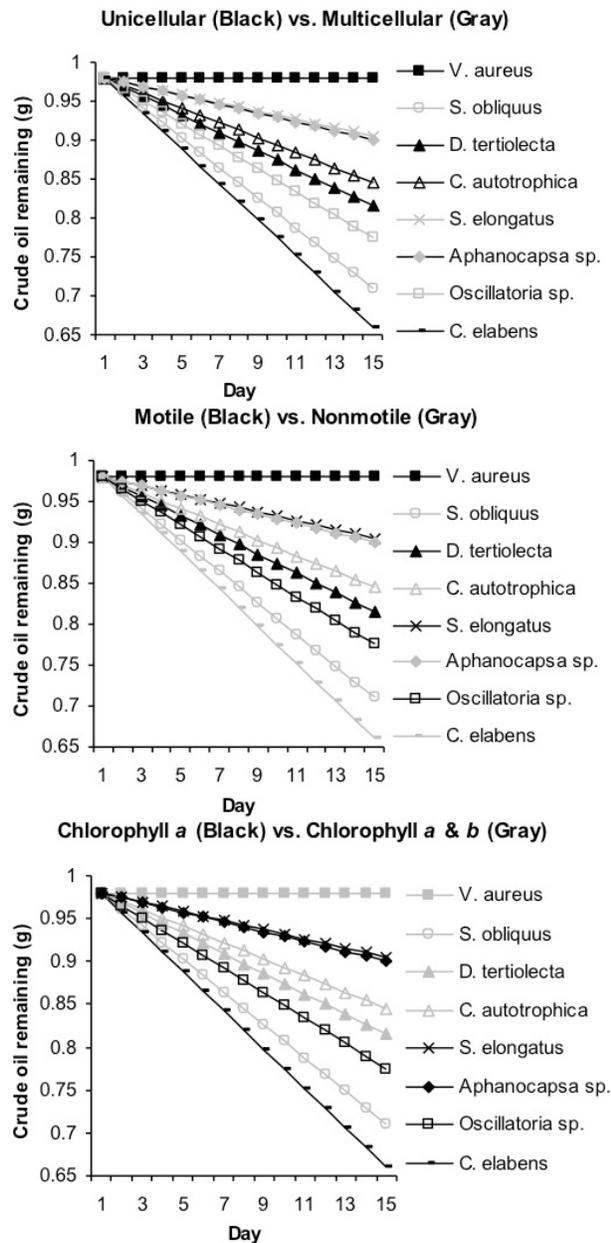


Figure 3. Graphs analyzed to determine trait to degradation rate correlation. No correlations were found as the top four degraders failed to exhibit a similar trait.

algal crude oil degradation rates. This result may be because the three characteristics tested do not affect degradation but a less obvious variable plays a role. Nevertheless, two significant conclusions were made through this research.

One important conclusion was that algae can degrade “heavy” crude oil, some of the most malignant portions of oil spills. “Heavy” crude oil, because of its viscosity and high burning point, is harder than its lighter counterpart to remove from spill sites; it often complicates pumping procedures and resists conflagration methods. Also, “heavy” crude oil contains harmful substances

like mercury and carcinogenic hydrocarbons such as naphthalene and benzo(a)pyrene. Fortunately, when issues involving the cleanup of “heavy” crude oil spills arise, we found that algae bioremediation can be presented as a plausible solution. The other conclusion was that algae could thrive in relatively high proportions of “heavy” crude oil with respect to the solution. The crude oil-to-solution ratio introduced to the tested algae was 1:13, compared to average oil spill ratios of 1:756,000,000 (1).

Even though the tested characteristics did not influence the algae bioremediation rate of crude oil in this research, this idea has potential; more characteristics can be analyzed with more trials. Also, a variety of crude oils can be tested. For method improvements, a gas chromatograph can be used to analyze the hydrocarbon composition of the Deepwater Horizon crude oil and all metabolites produced from degradation for more comprehensive results.

One next step for algae bioremediation is to ascertain a definite or best oxidation pathway that algae use to degrade hydrocarbons and then clone the cDNA for the specific enzyme that produces the best oxidation results. Once cloned, the gene can be over-expressed in algae via a proper over-expression vector through transfection. Also, genetic fortification would allow the enzyme to oxidize crude oil without an algal medium, eliminating the need for containment methods. The concept of algae bioremediation has much potential but requires further development.

One limitation of our project was not being consistent when administering the crude oil into the test tubes. Crude oil was measured using a one centimeter cube (with openings on 2 opposite sides) but this method was not very accurate in providing uniform amounts; this was a problem when we found that degradation occurred on the scale of thousandths of grams. Although none of the algae degraded all the oil, starting at minutely different amounts of crude oil made it inconsistent. Another limitation was the test tube environment. Though we used representative seawater and fresh water in the tubes, our environment was a rather crude simplification of a real biome, which has organism interactions.

In summary, the results from the study did not reveal a specific algal characteristic that increased specific algae bioremediation rate; however, it was found that *Coccochloris elabens* degraded nearly 35% of applied crude oil in 15 days, which should be further studied to determine the reasons for its high degradation rate. Overall, the hypotheses were not supported, but the investigation concluded that algae can efficiently degrade “heavy” crude oils and live in high “heavy” crude oil conditions, abilities that are necessary for crude oil bioremediation. Through this study, we have

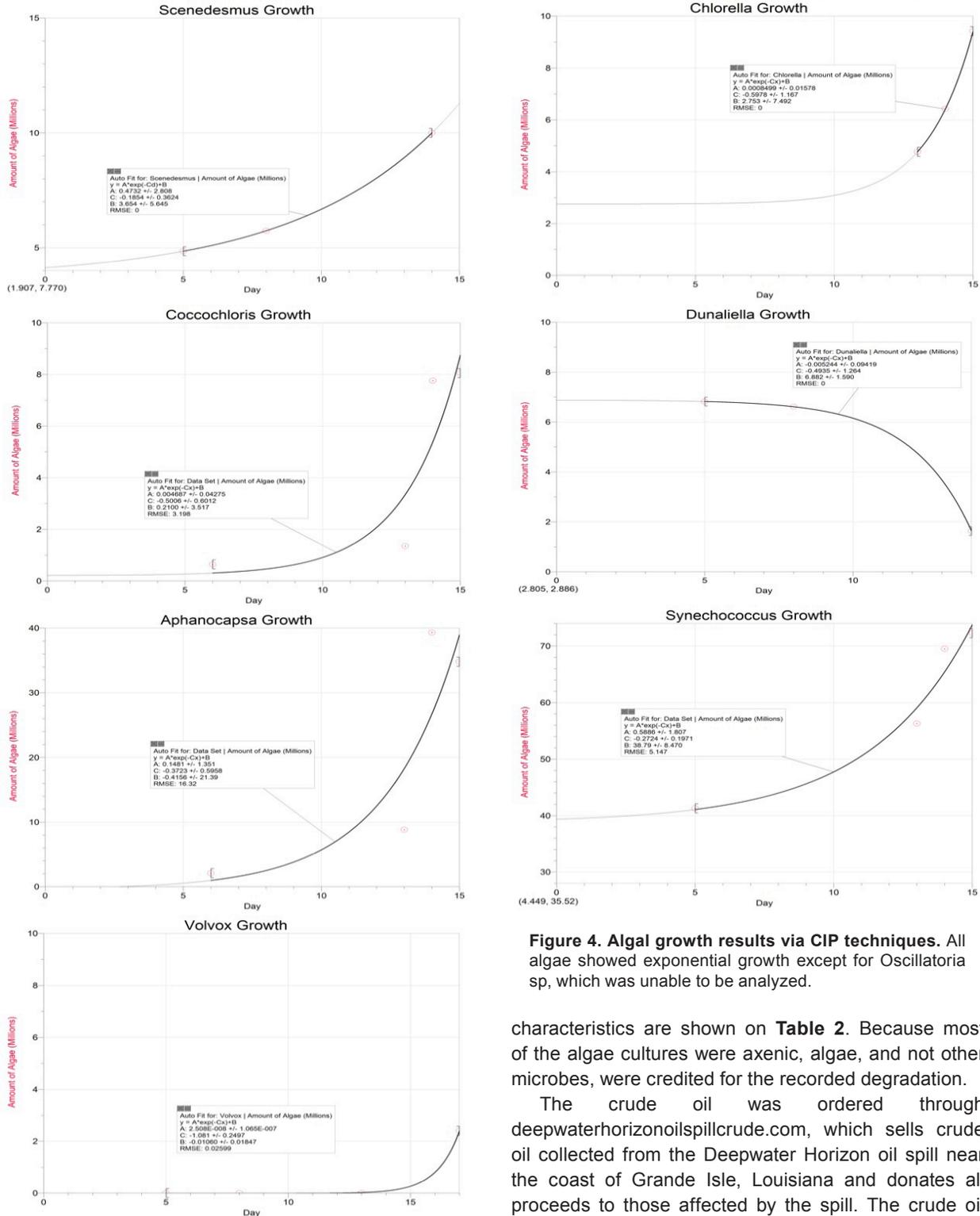


Figure 4. Algal growth results via CIP techniques. All algae showed exponential growth except for Oscillatoria sp, which was unable to be analyzed.

characteristics are shown on **Table 2**. Because most of the algae cultures were axenic, algae, and not other microbes, were credited for the recorded degradation.

The crude oil was ordered through deepwaterhorizonoilspillcrude.com, which sells crude oil collected from the Deepwater Horizon oil spill near the coast of Grande Isle, Louisiana and donates all proceeds to those affected by the spill. The crude oil was determined to be “heavy,” having a high density of 0.98 g/cm³ (determined by massing 1 cm³ of the crude oil). It was also viscous, adherent to all applied surfaces, and relatively solid at room temperature. Furthermore, the crude oil probably included heavy metals such as mercury, and lead, like most “heavy” crude oils (5).

demonstrated that algae are potential and efficient agents for crude oil degradation.

Methods

The algal sources abbreviations, species, and

Algae species	Motile	Chlorophyll Type	Cellularity
<i>Oscillatoria</i> sp.	yes	a	multi-cellular
<i>Synechococcus elongatus</i>	yes	a	unicellular
<i>Volvox aureus</i>	yes	a,b	multi-cellular
<i>Dunaliella tertiolecta</i>	yes	a,b	unicellular
<i>Aphanocapsa</i> sp.	no	a	multi-cellular
<i>Coccolithus elaberr</i>	no	a	unicellular
<i>Scenedesmus obliquus</i>	no	a,b	multi-cellular
<i>Chlorella autotrophica</i>	no	a,b	unicellular

Table 2. Algal species, species, motilities, chlorophyll types, and cellularity.

The project was initiated by administering 8 mL of Instant Ocean® seawater solution into six 15 mL marked test tubes (BD®) and 8 mL of freshwater solution (Ice Mountain® spring water) into two other test tubes. Five milliliters of *Volvox aureus* or *Scenedesmus obliquus* was applied with a Premiere® transfer pipette into one of the freshwater test tubes because they are freshwater algae. Five mL of the other six algae species were administered into the saltwater solutions with one species per tube. Then, the freshwater and seawater control experiments (lacking algae) were prepared, with 13 mL of the freshwater or saltwater solution. Afterwards, 1 mL of crude oil was added to all 10 test tubes. Due to its adhesiveness, the crude oil was difficult to measure and directly insert into the tubes. A method was introduced to transfer the oil from the original containers into the test tubes using aluminum foil. Crude oil was forced into a one-centimeter cube (with openings on 2 opposite sides) and extracted onto a 1.25 cm x 5 cm aluminum strip using a prod with a flat facet (smaller than the cube's sides). Lastly, this foil was rolled into a cylinder and placed in the tubes. Meticulous efforts and measurements allowed for 1 mL of crude oil per experiment.

The study was conducted in a Hamilton Safeaire® ventilated fume-hood (24±1°C) with constant light exposure by a General Electric® plant light. Saran® wrap was placed over the test tubes and three small holes were created over each of the tubes to allow aeration while limiting evaporation. To simulate oceanic tides, the test tubes were shaken for two minutes daily, preventing algae from accumulating at the bottom. Three trials were conducted for all tests, including the controls.

Mass, water decrease, and qualitative data were recorded for each test tube for 15 days. An Adventurer™ Ohaus digital scale recorded measurements in grams, and the mL indicators on the test tubes noted the water decrease. The rate of crude oil degradation was determined through the proposed equation:

$$\text{Rate of Crude Degradation} = \text{Avg}\left(\frac{\Delta m_{\text{test}}}{\Delta \text{day}}\right) - \text{Avg}\left(\frac{\Delta m_{\text{water}}}{\Delta \text{day}}\right) = \text{Avg}\left(\frac{\Delta m_{\text{crude}}}{\Delta \text{day}}\right)$$

This equation includes the only two changes in the experiments: mass and water amount (algal mass was determined to be insignificant through comparing the mass of a volume of distilled water to the mass of the

same volume of algae solution). The resulting difference between the total mass loss and the mass loss due to water can be attributed to algae degradation of the crude oil. A correlation between an algal characteristic and the rate of crude oil degradation would be present if the top four bioremediation algae exhibited the same characteristic. Lastly, t-tests through Microsoft Excel® were used to evaluate whether the algal test data was significant or due to random variability when compared to their corresponding control data. Comparisons were considered significant when p-values were under 0.05 for each algae test in comparison to the control tests, in which no algae was present.

At the end of each trial, all crude oil samples were extracted from the tubes and peeled from their aluminum shells to confirm their degradation results visually.

Algal growth was determined by computer image processing (CIP) techniques and spectrophotometry. The CIP techniques consisted of many steps. Based on the assumption that all the algae dispersed evenly throughout the solution, photos of a small sample of algae solution from each test tube on a slide were taken using a Zeiss™ “Axiovert” 40 CFL microscope every three days; the pictures were adjusted to a 32-bit black-and-white image via ImageJ™. Then the threshold was adjusted to isolate only the algae in the image. The algae for each test were counted per 0.0283 cm², and then algae quantities for 14 mL solutions were estimated. Through inserting a small sample of algae from each test tube into a ThermoScientific® Spectronic 20D+ spectrophotometer, the percent of light absorption (λ = 460 nm & 680 nm) was recorded before inserting the crude oil (first day) and after removing it (final day) for every test tube; then, the percent change in percent absorbance was concluded for each algae experiment.

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