

Impact of Surface Runoff-Derived Biodiesel on Marine Microbial Populations

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Abstract

The relationship between biodiesel production phases and the environment has been well studied; but little is known regarding biofuel interaction with the surface environment post-production (i.e. runoff, harbor spills). A laboratory-controlled experiment was performed to investigate the effects of biodiesel enrichment on near-shore seawater microorganism abundance. Over ten days, three sample replicates were analyzed for changes in biodiesel mass and cell density in coastal seawater collected from Kaimana Beach, Hawai'i. Methods of mass analysis and flow cytometry were used to identify variables.

Natural seawater with one percent added biodiesel exhibited (1) a 92% *increase* in cell density compared to a 65% *decrease* in cell density in the control without biodiesel, and (2) more reduction of biodiesel mass (80% elimination in natural seawater sample) over time compared to the filtered sterile seawater control (56% elimination). Although the second result is statistically less robust, these findings suggest that there is a relationship between biodiesel and microbial activity. The new direction in research based on these results should be a community DNA analysis to identify organisms utilizing the biofuel fatty acid compounds. These findings can be used in coastal management for spill or runoff mitigations and prevention of microorganism blooms thriving off biodiesel.

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Introduction

Anthropogenic inputs and the effects of urbanization can alter and disrupt natural biological community compositions, habitats, hydrologic systems, nutrient cycles and energy flow (Alberti et al 2007). The full extent of these disruptions remains unclear, regardless of whether the input is from a traditional or renewable energy source. Many researchers focus on developing new and more efficient renewable energy sources to overcome the dependence on unsustainable fossil fuels, yet the potential environmental interactions of any such renewable fuels have not been fully studied.

Near-shore runoff is generally associated with agricultural industry in rural environments (Puppán 2002). There are however studies that show the presence of high hydrocarbon and metal concentrations in runoff from urban environments near major highways and roads generated from motor vehicles. Soils near streets have been found to contain 250-1920 mg of hydrocarbon per kg of soil (Legret & Pagotto 1999). Furthermore, conventional fuel runoff around harbors, marinas, or from impervious terrestrial surfaces have various effects on marine microorganisms (Puppán 2002).

Although the impacts of agriculture and biofuel cultivation have been studied in depth, the impacts of post-production, post-combustion pollution of biofuel on hydrologic systems from runoff has significant environmental implications yet are poorly understood. This investigation studies the yet-unknown effects that biodiesel introduction has on marine microorganisms. The sampling location used for this study (as described in the methods section) was selected because it is impacted by urban run-off and is therefore a potential site of pollution by both conventional and

biofuel hydrocarbons. The goal of this project is to study the environmental effects that could occur from a terrestrial biodiesel spill and the impact that runoff can have on marine microorganisms.

Organism-specific reactions to biodiesel range from resistant or life inhibitory to stimulating (Moon *et al.* 2010). Microbes that can contaminate and survive in biofuels include sulfate reducing bacteria (SRB), aerobic bacteria, and some fungi (Benedict *et al.* 1996). Microbial productivity sustained by biofuels was found to be economically and environmentally useful for remediation purposes. While many studies have already indicated the negative effects of pollution created by agricultural production of biodiesel on the ecosystem (Uhlenbrook 2007), little is known regarding the environmental effects and interactions of the biodiesel. Studying the relationship between microorganism communities and biofuels like biodiesel is an important step to assess potential environmental impacts.

The previously discussed papers support the proposition that biodiesel produced by the chemical trans-esterification of fatty acids can potentially provide a metabolic carbon source for many microorganisms. Based on this, the following hypotheses were formulated:

- (1) Empirical Hypothesis 1: Biodiesel inputs into the marine environment can be naturally degraded by existing microbial populations.
- (2) Null Hypothesis 1: Biodiesel inputs into the marine environment cannot be naturally degraded by existing microbial populations.
- (3) Empirical Hypothesis 2: Biodiesel inputs into the marine environment will have a measurable impact on natural microbial populations.

- (4) Null Hypothesis 2: Biodiesel inputs into the marine environment will not have an impact on natural microbial populations.

Methods

The study will compare changes over time for both microbial cell population and biofuel reduction using (1) marine water samples and (2) a simulated hydrocarbon-polluted marine environment. *In situ* experimentation is unfeasible due to the variable marine environment (presence of other pollutants, changes in salinity, temperature, weather, variability of microbial communities would all affect community composition). Therefore, a benchtop experiment was performed with sampling over a ten day time period.

I. Sample Characterization

Samples of biodiesel concentration loss and microbial abundance were prepared for analysis. Biodiesel concentration data was taken gravimetrically (as described in Hydrocarbon Analysis below), and microbial abundance was determined through cell counts via flow cytometry (as described in Population Analysis below). To address natural variability, triplicates of each sample were analyzed. Beginning at day zero, each triplicate sample was sacrificed and analyzed once every three days, over a span of ten consecutive days.

A 12-L water sample was obtained from Kaimana's Beach on the southern end of O'ahu at 21°15'48" N, 157°49'17" W. This location was selected due to a lack of groundwater influx (Swarzenski et al 2013) so it is solely impacted by runoff. A YSI multiparameter water quality sonde device was used to measure initial temperature (23.88°C); dissolved oxygen (96.9 DO%), with 6.72 DO mg/L of water; salinity (34.37 Sal); and conductivity (52.18 mS/cm).

Triplicate water samples were dispersed into 40ml disposable polystyrene Falcon brand tissue culture flasks. Three triplicate separate sets were prepared (Table 1): Control 1 contained only 25 ml of natural seawater. Control 2 contained 25 mL of filtered sterilized seawater (0.22 μm filter) and approximately 1% w/v of commercially obtained biodiesel. The test sample contained 25 ml of natural seawater containing 1% w/v of biodiesel. The samples were placed in an incubated shaker set at 23°C rotating at 120 rpm.

Table 1. Each sample set and components

| Sample Set | Seawater | Biodiesel |
|-------------------|-----------------------|------------------|
| Control 1 | Natural | No |
| Control 2 | Filtered (sterilized) | Yes |
| Target | Natural | Yes |

II. Gravimetric Hydrocarbon Analysis

To quantify the biodiesel concentration, biodiesel gravimetric analysis was performed. On day zero, 0.25 mL of biofuel was added to the aliquoted 25 mL seawater for Test and Control 2 series. Over the experimental timeframe, biodiesel was extracted from the water samples through the gravimetric addition of approximately four milliliters of high performance liquid chromatography (HPLC) grade hexane solvent. The addition of the non-polar hexane solvent resulted in the formation of a biphasic solution that was easily separated from the water (Clark 2007). The non-polar hydrocarbon group is preferentially solubilized in the hexane and was easily extracted from water. Approximately two milliliters of the hexane and biodiesel mixture were then gravimetrically added to pre-weighed aluminum

pans and allowed to air dry in a hood for 3 days. The heavier biodiesel components have relatively low volatility and the residual product in the aluminum weigh boats represented the mass of biodiesel. After drying the pans were re-weighed.

The final and initial quantities of biodiesel were compared using the equations:

$$(1) \text{ Initial Biodiesel (g) / Total mL of mixture} = \text{Initial Biodiesel (g biodiesel/mL)}$$

$$(2) (\text{Mass of Dried Biodiesel \& Tin (g)}) - (\text{Mass of Tin (g)}) = \text{Residual Biodiesel (g)}$$

$$(3) \text{ Residual Biodiesel (g) / 2 mL} = \text{Final Product (g biodiesel/mL)}$$

III. Population Analysis: Flow Cytometry

Flow Cytometry was utilized to determine sample cell concentrations. Samples were prepared by fixing two milliliters of water samples using 50 μL of glutaraldehyde. Samples were frozen until used for analysis. Immediately before analysis, the samples were thawed and dyed with a Hoescht UV DNA dye. Viable cells at the time of fixation were then identified by flow cytometry. An Altra flow cytometer was used to determine cell counts.

Results

I. Hydrocarbon Results

Biodiesel mass loss from both control and inoculated samples are shown in Figure 1 and Table 2. Colored markers indicate sample means, and the error bars represent the standard deviations for the triplicate samples. The reduction in biodiesel mass in the sterile set (Control 2) is less than the reduction of biodiesel

mass in the inoculated Target samples. Thus, the microbiological consortia do visibly appear to have degraded the biodiesel faster than with chemical oxidation only and to a further extent over the time period of the study. After 10 days, the biodiesel in the seawater sample (Target) was degraded by 80%, from 0.025 g biofuel/mL to 4.92×10^{-3} g biofuel/mL. In the sterilized seawater sample (Control 2), the biodiesel mass was reduced by only 56%, ending at 1.07×10^{-2} g biofuel/mL.

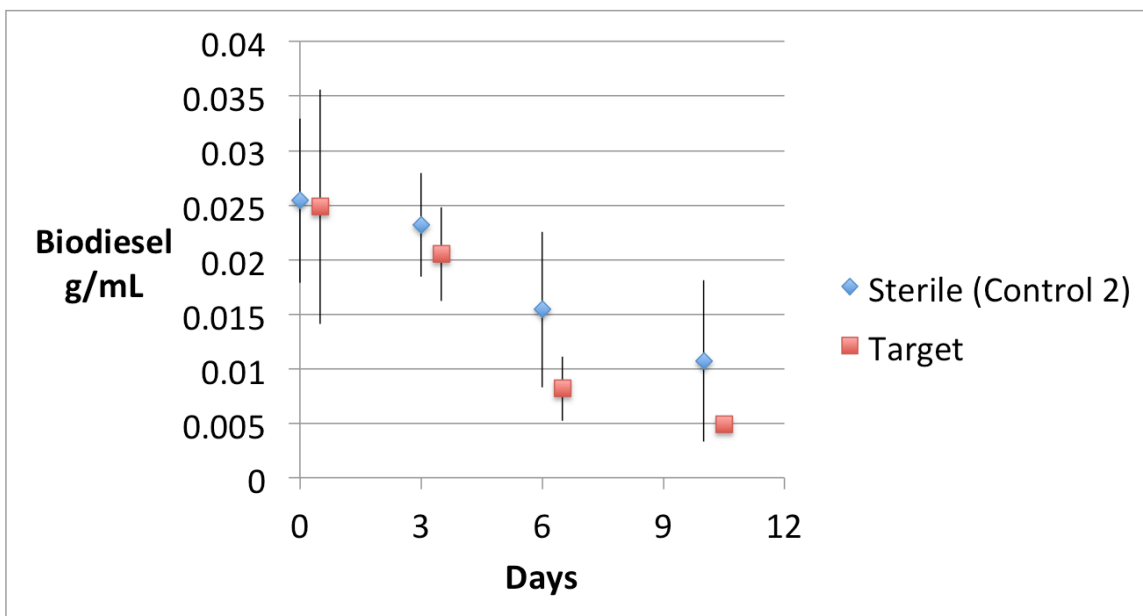


Figure 1. The average biodiesel mass decreases over time in both the Control set (Control 2 = sterile seawater + biodiesel), and the Target set (natural seawater + biodiesel). Error bars display the standard deviation of the triplicates at each day. The Target sample on day 10 was not analyzed in triplicate so the standard deviation is not included.

* The Target data set was shifted by +0.5 days along the x-axis for clarity of

II. Statistical Analysis of Hydrocarbon Results

Since there is significant overlap in the sample standard deviations (error bars shown in Figure 1), for the sterile Control and Target biodiesel masses, a further statistical analysis was done (Student's *t*-test) to clarify the statistical significance of results.

| | Day | Biofuel Triplicate (g/mL) | Sample Mean | Sample Standard Deviation | |
|--------------------------------|-----|------------------------------|-------------|---------------------------|-----|
| Control 2 (Sterile) | 0 | 0.0333 | 0.0254 | 0.0075 | |
| | | 0.0247 | | | |
| | | 0.0183 | | | |
| | 3 | 0.0279 | 0.0232 | 0.0048 | |
| | | 0.0233 | | | |
| | | 0.0183 | | | |
| | 6 | 0.0101 | 0.0154 | 0.0071 | |
| | | 0.0126 | | | |
| | | 0.0236 | | | |
| | 10 | 0.0115 | 0.0107 | 0.0074 | |
| | | 0.0177 | | | |
| | | 0.0029 | | | |
| Target | 0 | 0.0368 | 0.0249 | 0.0107 | |
| | | 0.0218 | | | |
| | | 0.0159 | | | |
| | 3 | 0.0166 | 0.0205 | 0.0043 | |
| | | 0.0251 | | | |
| | | 0.0198 | | | |
| | 6 | 0.0061 | 0.0082 | 0.0030 | |
| | | 0.0069 | | | |
| | | 0.0116 | | | |
| | * | 10 | 0.0049 | 0.0049 | N/A |

Table 2. Triplicate data of results from Control 2 and Target sets, data also shown in Figure 1.

* Two samples of day ten were physically obstructed within the lab.

| Day | Difference Between Means Control 2 - Target | Standard Deviation of the difference between means (1) | t-statistic (2) | t-critical (95%), 4 degrees of freedom | t-critical (80%), 4 degrees of freedom |
|-----|--|---|-----------------|--|--|
| 0 | 0.0006 | 0.0076 | 0.08 | 2.78 | 1.53 |
| 3 | 0.0027 | 0.0037 | 0.72 | 2.78 | 1.53 |
| 6 | 0.0073 | 0.0045 | 1.62 | 2.78 | 1.53 |
| 10 | 0.0058 | N/A | N/A | N/A | N/A |

Table 3. A statistical analysis of residual biofuel derived from the difference between means of Control 2 and the Target. Declaring statistical significance when t-statistic > t-critical.

(1) Wessel 2009, Eqns. 2.6, 2.7

(2) Wessel 2009, Eqn. 2.8

Table 3 shows the difference between the sample and control mean is 6×10^{-4} , and the standard deviation of that difference is one magnitude larger than the difference value. The lower t -statistic values (Column 4, Table 3) than the t -critical values (Column 5 & 6), suggests that the null hypothesis cannot be rejected with 95% confidence. Only the difference on day six is significant at 80% confidence level. A sample from day ten was lost and a triplicate was unable to provide a mean and standard deviation. It cannot be argued that the sample means are statistically different at the 95% significance level because the sample t -statistics do not exceed the critical t -statistic values shown in the fifth column of Table 3.

The statistical analysis displays that **Null Hypothesis 1 cannot be certainly rejected**, even though the target sample was observed with less residual biofuel than the sterile sample at the end of the experiment. Due to the small concentration of residual biodiesel and low number of replicate samples, the observed difference was not statistically distinct enough to determine statistical significance. The trend in differences in reduction is displayed in Figure 2 where residual biofuel from Control 2 was subtracted from the Target set to show where the major observed differences occurred. The error bars in Figure 2 displays the statistical uncertainty of the real differences in biodiesel reduction between the Target set and Control 2. The differences in reduction are too small for accurate detection via gravimetric analysis, which is why Null Hypothesis 1 cannot be rejected using these methods.

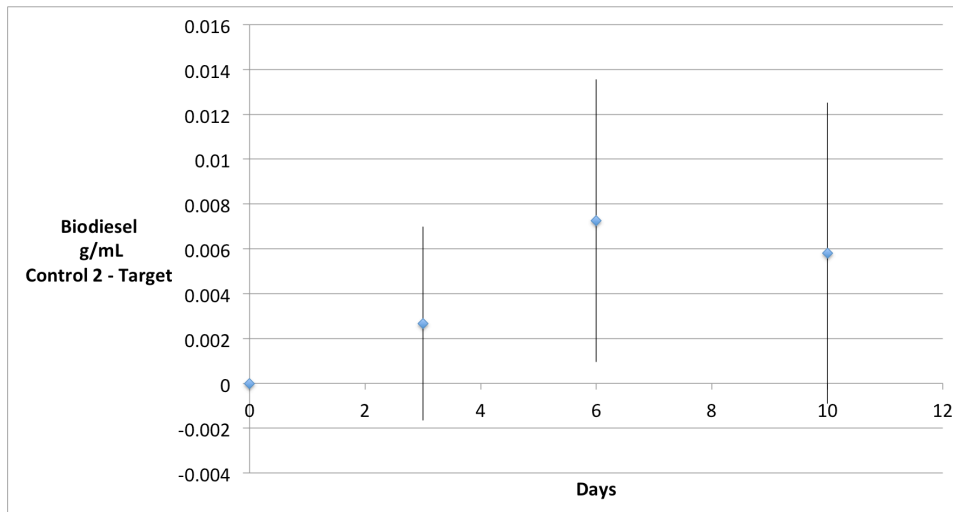


Figure 2. Natural seawater influenced reduction overtime. The figure displays the differences in biofuel reduction between Target and Sterile Control 2.

III. Population Results

Changes in microorganism cell numbers are presented in Figure 3 using data from flow cytometry. Figure 3 displays the changes in the cell density for the natural seawater (Control 1) and the test sample. Cell counts in the biodiesel seawater sample increased by 92%; 612,000 cells/mL compared with sterile samples that decreased in cell count by 65%. Standard deviation error bars are too insignificant to be plotted on the graphical scale used. The significant difference in the mean values and standard deviation, allows for **a rejection of the second Null Hypothesis**. The presence of biofuel did have an influence on marine microbial population. The natural seawater control that did not contain biodiesel had a rapid reduction in cell numbers within the closed system. Furthermore, while statistical significance was not achieved for the reduction of biodiesel when compared with controls, the increase in cell numbers correlate well with the reduction of biodiesel displayed in Figure 1. The largest increase in cell populations correlates with the

largest difference of biofuel reduction (Figure2) on day three. The increase in control populations on day three could be due to stimulations during acclimatization of organisms to the test conditions, but could not be sustained after those resources were utilized. Overall, the natural seawater and biofuel mixture of the Target set provided ideal conditions for microbial growth regardless of the fact that it was a closed system.

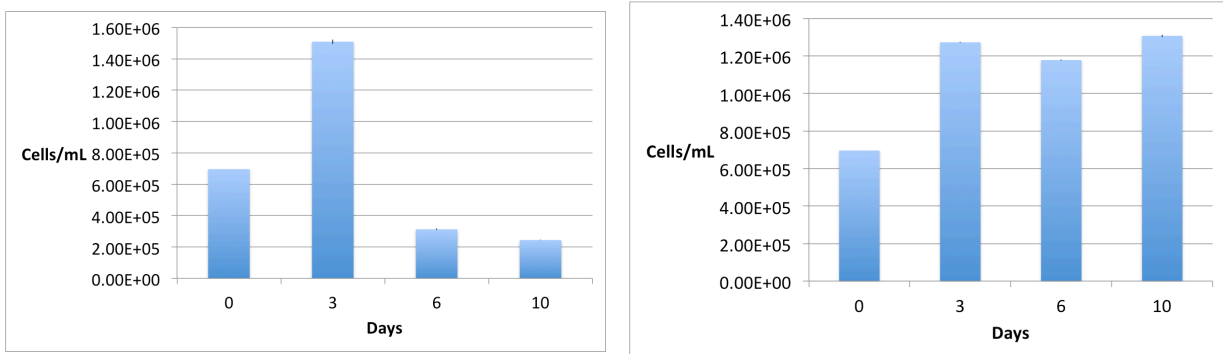


Figure 3. Cell counts for Control 1 (left) and Target set (right).

Discussion

I. Statistical Significance vs Observation

Although the statistical significance tests cannot reject Null Hypothesis 1, they do reject Null Hypothesis 2. To tie in the two hypotheses; the cell population was positively influenced by the presence of biofuel, but the biofuel was not certainly influenced by the presence of microbial activity. It was initially thought that these two hypotheses were mutually dependent upon each other; even with the statistical insignificance of the first hypothesis, the results could still be interpreted that biodiesel provides a bioavailable source of carbon that can support cell growth.

The combined observation of microorganism growth and biofuel reduction calls for further investigation to verify if the microorganisms were the influencing factor on the additional biodiesel difference (Figure 2). Biodiesel stability may be influenced by many factors, but a more intricate analysis will be required to attribute enhanced biodiesel reduction to the microbiological consortium. This result also shows the need for a better method to determine biodiesel mass loss. Instead of a gravimetric analysis of the residual biofuel, a mass spectrometer could provide more accurate results and enhance the data set by determining what specific hydrocarbon fractions were metabolically utilized. This information could also provide insight into differences between chemical oxidation and biological degradation. Furthermore, error could have been introduced through the introduction of seawater that may have been incorporated into the hexane extraction. A mass spectrometer would also allow better efficiency with sample analysis, more replications of sets, and less error. Both errors described would be eliminated with use of a mass spectrometer.

II. Broader Impacts

The doubling of observed cell numbers in the target sample can be attributed to the addition of biofuel. The cell counts from flow cytometry do allow for some reasonable broader impacts. The large increase in microorganism concentration poses some environmental implications. Biodiesel is constructed using natural sources of fatty acids that can be metabolized by a variety of biological organisms. Biodiesel is a chemically synthesized compound that utilizes fatty acid compounds. Heterotrophic metabolisms allow for the catabolic utilization of fatty acids through beta-oxidation (Vyas et al 2010). The provision of a carbon source in an open

system like near-shore marine environments can have similar impacts to what was experimentally observed.

Although the majority of biodiesel that was added to the sample was degraded within ten days, that rate may be very different in the natural environment, and the effects on other trophic levels might be different. This investigation suggests that there are natural populations of microorganisms in seawater that will digest biodiesel. Degradation may be affected and facilitated by the physical pathways that a contaminant takes. Urban pathway patterns highly influence runoff and bioremediation and biogeochemical processes (Alberti 2007). For example the degradation period may also be reduced if the biofuel spilled into a marina, and had greater surface dispersion increasing biological interactions.

As microbes consume and grow from pollution sources, the trophic balance will shift leaving fewer resources, like oxygen, for the rest of the community of that environment. Other sources of anthropogenic pollution of nutrient overload are known to increase algal and microorganism blooms, thereby creating zones of low oxygen and more acidic waters where loss of marine habitat and biodiversity are common (Diaz 2003, Brasher & Wolff 2007). Expanding oxygen minimum zones are a new phenomenon of anthropogenic development studies, but they are known to alter marine biogeochemical processes along with stressing the ecosystem with hypoxic waters (Stramma et al 2008). If a microbial population in a near-shore environment were to double due to nutrient pollution, the oxic conditions would turn hypoxic or even anoxic if the system was not replenished with the oxygen utilized by the new doubled population. Hypoxic conditions would essentially redirect the biogeochemical interactions and decay that doubled population, along

with other microorganisms and further up the trophic spectrum as anoxia increased. Cell populations thriving off biodiesel runoff could pose implications of anoxic conditions in runoff-influenced bodies of water. Yet, the impacts of anoxia are relatively low compared to that of a conventional diesel spill or runoff, which requires anthropogenic mixtures of surfactants to deteriorate the oil flux to the environment since natural microbial activity is unable to quickly break down crude oil contamination. In addition, the potential for natural remediation of biodiesel spills makes biodiesel a more environmental friendly option of conventional fuels.

Conclusion

This study observes that during simulated seawater contamination with biodiesel, marine microorganisms doubled in population and used biodiesel as an energy source. This suggests that runoff containing biodiesel would be naturally remediated but simultaneously would potentially cause environmental impacts as a significant increase in microbial composition may affect oxygen content of the water. Biodiesel spills therefore may require similar considerations to other terrestrial anthropogenic inputs that can make their way into the marine environment. Within the short time span of just ten days, the majority of biodiesel in the closed experimental conditions was eliminated by 80%. Based on the observations of this investigation, it is clear that the effects of a microorganism bloom should be taken into consideration regarding biofuel pollution. Further DNA sequencing of the cell populations analyzed in the flow cytometer would advance our understanding of the role of microbial biodegradation in case of biofuel contamination remediation. The specific magnitudes of biofuel-environmental

interaction may vary, but more statistically sound data would come with a more in depth simulation regarding biodiesel reduction through the use of mass spectrometry and DNA sequencing analysis to verify if specific organisms utilized the biodiesel as sustenance during the study. This study has shown that such avenues of research have a great potential.

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