Final Report to the Louisiana Sea Grant
Undergraduate Research Opportunities Program

Hydrocarbon Bioconcentration by *Rangia cuneata* at Varied Salinity

Katherine Parenteau and Phillip Voegel
Department of Chemistry & Physics
Southeastern Louisiana University
Hammond, LA 70402

Introduction

The Deep Horizon oil spill has become known as one of the worst spills in history. It pumped oil into the gulf at a rate of 2.5 million gallons a day from April 20-July 15, 2010. This created a spill of over 245 million gallons of oil (NOAA, 2010a; NOAA 2010b). As each containment measure failed, there was increasing national attention on clean-up and recovery for the fragile Gulf coast ecosystems.

Several clean-up methods were used, each with its own strengths and environmental consequences. Some oil was burned, while that removed the oil from the water, it created its own pollution source. Skimming the water surface was attempted, but proved to be fairly ineffective. Only 3% of the spill was cleaned by skimming (MacKenzie, 2011). One of the most debated methods used was chemical dispersants. These chemicals break oil into smaller droplets. Because of the breakdown there were no pictures of oil-covered bird; but the oil was more digestible by marine life. The droplets also mixed more readily into shallow water and mud. With these anaerobic conditions it can take decades longer to degrade than if it had remained on the surface. Limited data is available about the possible toxicity of the dispersants themselves (Zuijdgeest, 2011).

Another option gaining ground in clean-up methods is bioremediation: the use of organisms to either remove or degrade the oil in the water. Many bioremediation studies focus on the use of bacteria to break down the oil. This study examines the use of *Rangia* clams for the removal of hydrocarbons. *Rangia* clams are an abundant species in Lake Maurepas and Lake Pontchartrain. Many studies have been done regarding filter feeders, like *Rangia cuneata*, being contaminated during the spill when they filter hydrocarbons from the water. This study will exploit that ability in order to remove the hydrocarbons from lake water.

Project Objectives

1. Set up control and experimental tanks. Each will be filled with lake water and 50 ppm hydrocarbons (hexadecane) will be added. The clams will be added to the experimental tank. Samples of water for hydrocarbon analysis will be taken every hour for five hours. Previous experiments have shown that *Rangia* clams completely remove up to 50 ppm of simple hydrocarbons under the laboratory conditions that will be employed. The salinity of the water will be recorded. This will allow us to set the baseline for removal of hydrocarbons.

This portion of the project was successfully completed two times. The first trial was completed during the spring 2014 semester prior to funding of the proposal while a second trial was completed during the fall 2014 semester.

2. For the next experimental setup the salinity in the tanks will be adjusted to various levels to mimic saltwater intrusion that is common during storm seasons. These conditions were encountered during the Deep Horizon spill. This will also help to determine if the clams can be used in water that is more brackish due to its location.

   Experiments at varied salinity were not completed. Katherine held an internship position at Motiva in Baton Rouge, LA during the summer and did not begin the project in earnest until August 2014. At this time, medical issues that had been under control reemerged and limited the amount of laboratory work that she was able to complete.

3. The data will be analyzed to determine if salinity is a limiting factor on being able to use *Rangia* clams to remove hydrocarbons. The simple and polycyclic aromatic hydrocarbon levels will be measured separately to determine if there is any difference in the removal of the two types as salinity changes. I will prepare a final report for my professor and at least one conference presentation. With the assistance of my professor, I also will prepare a manuscript for publication in a scientific journal.

   This goal has been partially met. Katherine prepared abstracts for presentations at two local conferences – a student poster session with the Louisiana Local Section of the American Chemical Society held at Xavier University in October 2014 and an undergraduate research conference held at LSU in October 2014. However, for health reasons, she did not attend either conference. Due to the limited nature of the data Katherine has provided, it is unlikely that a manuscript can be prepared for publication at this time.

Methods

Water & Clam Collection: Water samples and live *Rangia* clams were collected on April 4, 2014 and August 25, 2014 from the western end of Pass Manchac in Lake Maurepas (30° 16.453' N x 90° 24.527' W, Figure 1). The site was located using a Garmin eTrex handheld GPS and reached by boat. Approximately 15 gallons of lake water were collected by dipping three five gallon buckets into the lake. Five medium sized clams, about 2 inches in diameter, were collected by running a dip net through the silt lake bottom. The clams were separated from the silt prior to placement into one of the five gallon buckets for transport and storage.

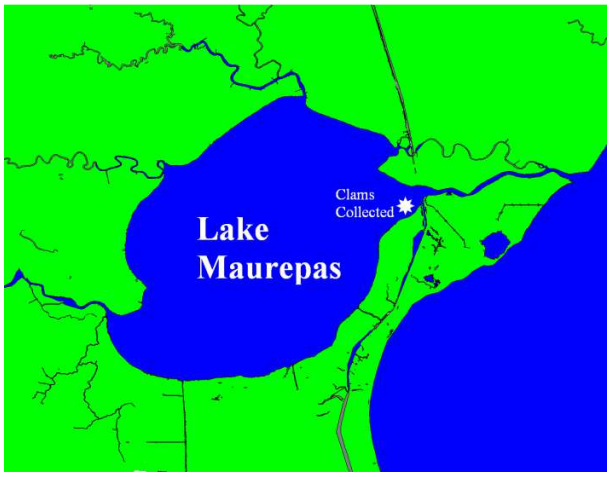

Figure 1. Lake Maurepas site for collection of water and clams.

Tank Setup: Immediately upon return the laboratory at Southeastern Louisiana University, the lake water was used to fill two five gallon aquariums and the over-tank filter was filled with water, primed, and started. The filters were used only to provide circulation and aeration and did not contain any filtration materials. The clams were placed in one of the aquariums (experimental tank) while the other tank contained only lake water (control tank). The tanks were allowed to equilibrate to laboratory conditions for 24 hours.

Hydrocarbon Addition and Laboratory Sample Collection: 1.22 ml of hexadecane were added to the experimental and control tanks providing an initial hexadecane concentration of 50 ppm. After 30 minutes, a set of three 100 ml samples were collected from each tank. This process was repeated once an hour for a total of five samples.

Extraction: The hexadecane was extracted from each water sample using tetrachloroethylene with a separatory funnel. Individual samples were treated three times each with 3.00 ml of tetrachloroethylene. The tetrachloroethylene extracts for each individual sample were combined in a 10 ml volumetric flask. After the addition of decane as an internal standard to each volumetric flask, the flasks were filled to the mark with tetrachlroethylene. The extraction method is based on Standard Methods 5520C (Clesceir, 2005) with exchange of trichlorotrifluoroethane with tetrachloroethylene because trichlorotrifluorethane has been banned as an ozone depleting substance. Several literature sources as well as previous work in this lab have demonstrated the effectiveness of this solvent in place of the banned substance (Farmaki, 2005; Farmaki 2007; Miralles, 2007). The extracted samples were analyzed by gas chromatography/mass spectrometry to verify that other hydrocarbons were not present and the concentration of hexadecane determined by gas chromatography with a flame ionization detector. Since the 100ml of water taken five times throughout each trial represent only about 2% of the total volume of the aquariums, their removal is not expected to affect the functioning of the system.

***GC/MS Analysis*:** GC/MS analysis of tetrachloroethylene extracted water samples was completed using EPA Method 8015. A Varian 450-GC equipped with a Varian 220 MSD and a 30 m x 0.25 mm DB5-ms column was employed for the analysis of 1 µl extract injections. The initial temperature in the GC oven was 45°C and was held for 2 minutes after injection. The temperature was then increased at a rate of 12°C per minute to a final temperature of 212°C.

The final temperature was held for 0.8 minutes for a total run time of 16 minutes. The mass spectral detector remained inactive until 6.5 minutes into the run to allow the tetrachloroethylene solvent to pass through the system. The isotopic abundances and the fragmentation patterns of each peak observed were analyzed to verify that only decane and hexadecane were present in the extracts.

GC/FID Analysis: After verifying that only decane and hexadecane were present in the samples, the concentration of hexadecane in the extracts was determined using the internal standard method by comparison to standard solutions prepared in the same manner as the extracts. This analysis was completed using an Agilent 6890N gas chromatograph equipped with a flame ionization detector and an Agilent 7683 series autosampler. The oven program and column specifications were the same as those used in the GC/MS analysis. Using the GC/FID instead of the GC/MS for the determination of hexadecane concentration was advantageous because scheduling conflicts with other students and faculty in the department were avoided and because the ability to use the autosampler made completion of the analyses much more efficient.

Results and Discussion

The first step in this research was to verify that hexadecane was extracted from aqueous samples as expected using tetrachloroethylene. A standard solution of hexadecane containing decane as an internal standard was injected into the GC/MS to determine the retention times of the two compounds and to obtain the mass spectra of hexadecane for comparison to any peaks observed in samples extracted from the aquariums. The chromatogram obtained for a representative injection of hexadecane standard is shown in Figure 2 and a retention time of 11.28 minutes. Comparable retention times were observed for a peak in the samples extracted from the control tank (Figure 2B) and the experimental tank (Figure 2C). An additional small peak that was not present in the standard solution is evident in samples extracted from both the control and experimental tanks. For the specific chromatograms shown in figures 2B and 2C, the ratios of the signal of hexadecane to decane are 9.10 and 4.70, respectively, demonstrating that the concentration of hexadecane in the experimental tank containing clams is much lower than in the control tank.

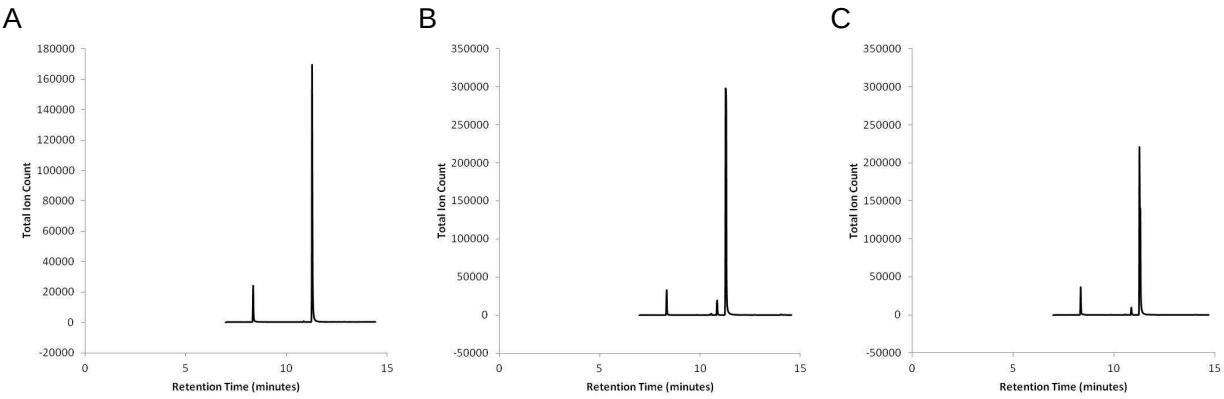

Figure 2. GC/MS of 1µL injection of (A) hexadecane standard prepared in tetrachloroethylene with decane internal standard, (B) tetrachloroethylene extracted water sample from the control tank taken 30 minutes after addition of hexadecane and (C) tetrachloroethylene extracted water sample from the experimental tank taken 30 minutes after addition of hexadecane

To further verify the identity of the hexadecane peak in samples extracted from the control and experimental tanks, the mass spectrum of the peak at 11.28 minutes was obtained and compared to that of the hexadecane standard. Figure 3 shows the mass spectrum of hexadecane in the standard (Figure 3A) and from the sample extracted from the experimental tank 30 minutes after addition of the hydrocarbon (Figure 3B). While the expected parent ion peak at m/z = 226 for hexadecane was not observed in either spectra, the same fragmentation is clearly obtained for both samples. Major fragment ion groupings are observed at m/z = 43, 57, 70, 85, 99, 111, 125, 140, 154, and 168 were consistently observed from GC/MS experiments using hexadecane from standard solutions, extracted from the control tanks, and extracted from the experimental tanks.

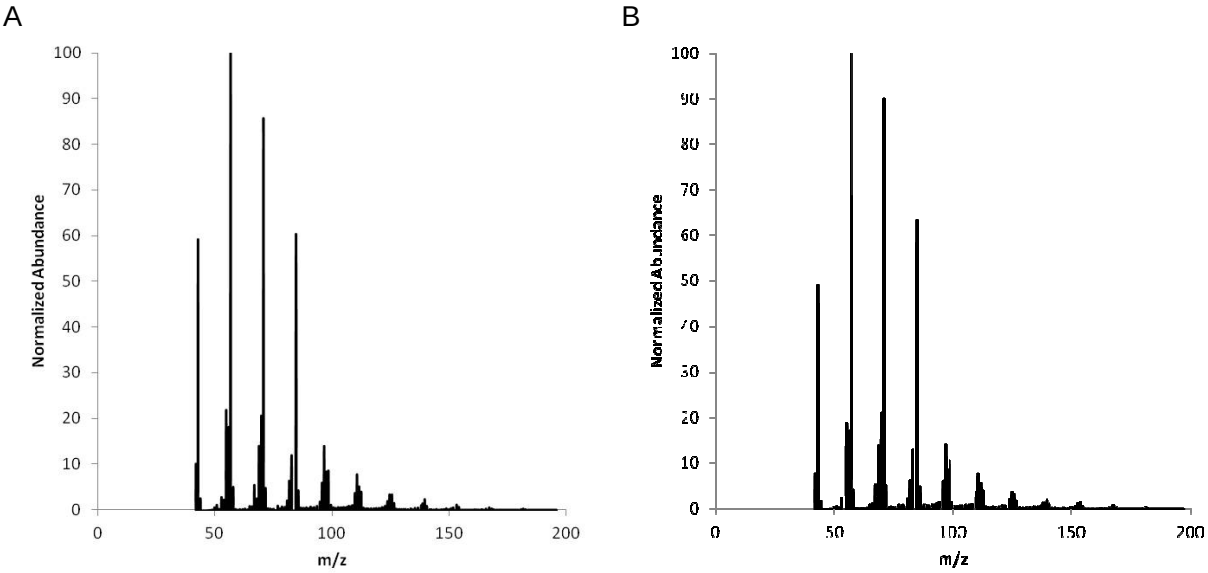

Figure 3. Mass spectra for hexadecane from (A) a standard solution prepared directly in tetrachloroethylene and (B) extracted from an experimental tank containing clams.

Once the identities of the major peaks in the GC/MS chromatograms were confirmed to be hexadecane and the internal standard decane, the remaining analyses were to be completed using GC/FID. This instrumental was newly obtained in the Department of Chemistry & Physics at Southeastern at the beginning of the fall 2014 semester and included an autosampler. This instrument replaced an aging GC/FID that was housed in Dr. Voegel's laboratory and had been the intended instrument for use in this project. Using GC/FID instead of GC/MS does not provide structural information and confirmation of peak identity for each trial; however, multiple researchers in the department use the GC/MS on a regular basis and this limits the number of samples that can be analyzed in a timely fashion using this instrument. No other research groups consistently use the GC/FID making it a much more convenient instrument for collection of the large amount of data associated with this project. Representative chromatograms recorded by GC/FID are shown in figure 4 below. Included in this figure are four chromatograms of samples collected from both control and experimental tanks at 0.5 hours and 4 hours after hexadecane was added to the tanks. To avoid the extremely large signal

associated with the tetrachloroethylene solvent, the FID signal has been truncated to exclude all signals recorded prior to 3.5 minutes. Additionally, the signal illustrated in these chromatograms are normalized as the ratio of the FID current at any given retention time to the maximum current observed for the decane peak located at 5.0 minutes in each chromatogram.

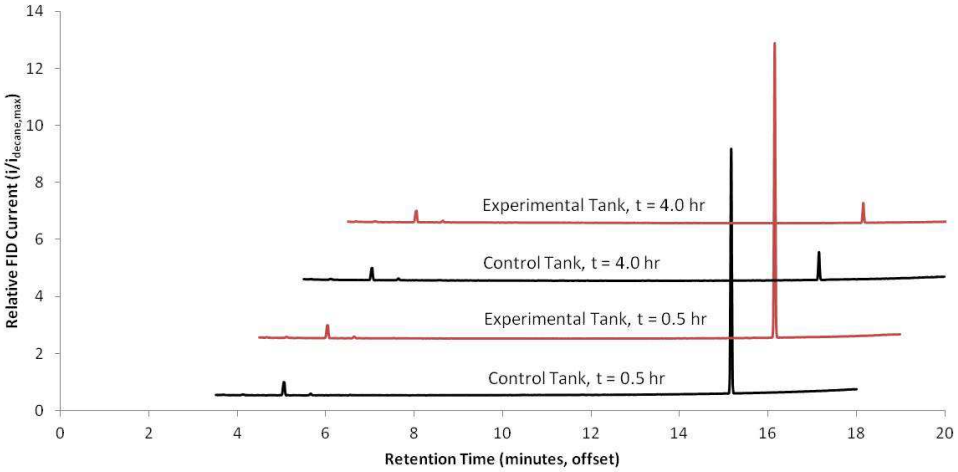

Figure 4. GC/FID results for hexadecane extracted from experimental and control tanks at 0.5 and 4 hours after addition of hexadecane to the tanks.

As seen in figure 4, the concentration of hexadecane is much smaller in both the experimental and control tanks four hours after its addition to the tanks. In these specific chromatograms, the concentration of hexadecane has decreased by 88.3% at 4.0 hours compared to its concentration 30 minutes after addition to the experimental tank and by 83.1% over the same timeframe in the control tank.

The GC/FID data above is illustrative of the type of data obtained for each replicate for each extraction completed during two complete trials of this experiment. On two separate occasions during 2014, clams were collected from Lake Maurepas and placed in 5 gallons of lake water in the experimental tank while 5 gallons of lake water were added to a separate control tank without clams. After a 24 hour equilibration period, hexadecane was added to each tank to an initial concentration of 50 ppm. Thirty minutes after addition of hexadecane, three 100 ml samples of water were collected from each tank, extracted into tetrachloroethylene as describe above, and analyzed by GC/FID. The collection and extraction process was repeated one, two, three, and four hours after addition of hexadecane. A total of 15 samples from each control tank and 15 samples from each experimental tank were analyzed in triplicate by GC/FID for each set of clams collected from the lake. The ratio of hexadecane to decane for each replicate sample extracted at each time following extraction was calculated by comparison to a calibration curve developed from standard solutions prepared in tetrachloroethylene. The average concentration of hexadecane in each tank at each time after addition of hexadecane was then calculated and compared. Figure 5A shows the calibration data collected for standard solutions for the final trial of the experiment completed during the fall 2014 semester. Linear regression for the calibration data determined that the slope was 0.0677 ppm^{-1} with an intercept of -0.015 and a

correlation of determination (R^2) of 0.999. Using this calibration data, the concentration of hexadecane in each sample was determined and their average values are shown in figure 5B for each sample collection time after addition of hexadecane to the experimental and control tanks.

A B

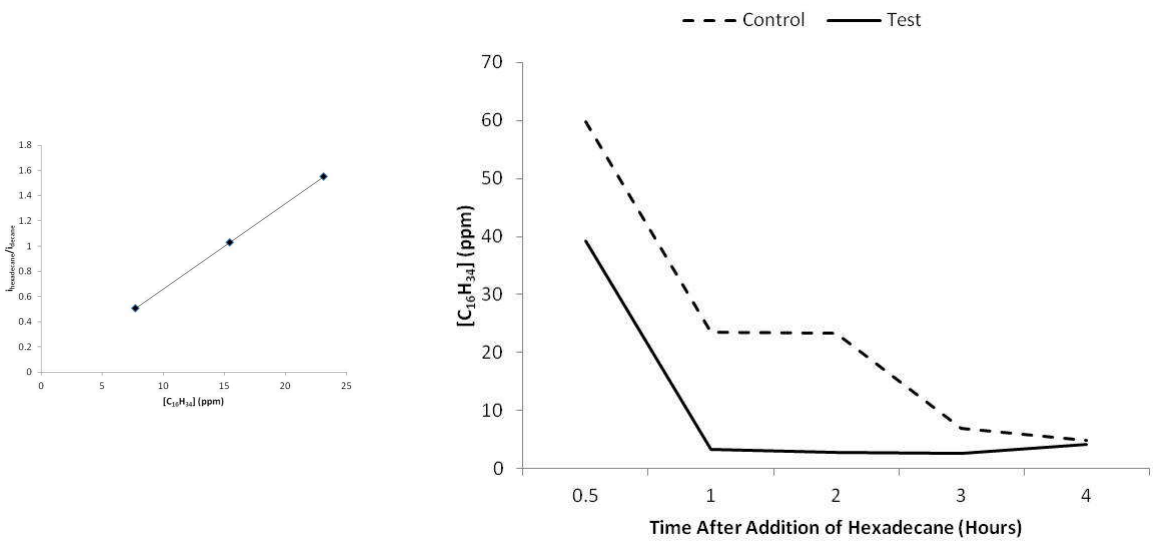

Figure 5. (A) FID signal ratio for hexadecane calibration standards and (B) concentration of hexadecane in experimental and control tanks over time.

Conceptually, the project has been successful in that when hexadecane was added to experimental tanks containing clams from two separate collection trips, the concentration of hexadecane was observed to decrease more rapidly than in tanks containing no clams. The most significant problem observed in this research was inconsistency in the extraction of hexadecane from the water samples leading to large differences in hexadecane concentration within replicate samples. For this project to be more successful in the future and to be applied to water samples of different salinities as planned, a more consistent extraction method must be developed.

Budget

The funds allocated for supplies were expended essentially as planned. Items purchased included tetrachloroethylene ($120), dichloromethane ($113), separatory funnels ($345), a gas chromatography column ($410), and caps for autosampler vials ($65) . Within this category, 91% of funds were expended. The amount spent on separatory funnels was significantly higher than budgeted ($110) and caps for autosampler vials had not been included in the original budget because the use of an autosampler had not been available at the time the proposal was submitted. However these increases in spending were offset by the purchase of the nitrogen regulator in the original budget ($365) by Southeastern's Department of Chemistry & Physics for the project after submission of the proposal but prior to funding and the actual cost of the

chromatography column was somewhat less than budgeted ($470). Finally, unexpected health problems led to limited effort on this project by Ms. Parenteau and, for this reason, she chose not to accept payment of hourly wages for the project and thus none of the $1000 budgeted for wages for the project were expended. Additionally, health issues prevented Ms. Parenteau from presenting her preliminary results at two undergraduate research conferences as planned and the budget for travel was also not expended. Despite the limited effort, earlier preliminary results have been confirmed demonstrating the potential for the removal of hydrocarbons from natural surface water systems by *Rangia* clams.

Conclusion

Despite limited effort by the student, preliminary results obtained by a previous undergraduate research student in Dr. Voegel's laboratory have been confirmed. Rapid removal of a model hydrocarbon, hexadecane, by *Rangia* clams was demonstrated with nearly 90% of the compound removed from the experimental tank within 4 hours. Future efforts in this line of research will focus on several objectives. (1) The extraction procedure will be practiced and improved to increase the consistency of data obtained for the project. (2) The salinity of the water in the aquariums will be adjusted to determine if significant differences in the removal of model hydrocarbons occur. (3) Additional aliquots of model hydrocarbon will be added to the experimental tanks to determine the extent to which bioremoval of hydrocarbons by *Rangia* clams can be continued. (4) The clams will be placed in clean water to determine if the hydrocarbons will be released back into water thus allowing the clams to be safely returned to their natural habitat following cleanup of a spill.

References

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