

Title: Bacterial crude oil bioremediation across different sediment systems.

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Introduction

In 2024, there were 6 large oil spills across the world, releasing approximately 7 tons of crude oil, permanently affecting ecosystems across the world (Oil Tanker Spill Statistics 2024). Crude oil, in excess, can be extremely harmful to animals' aquatic ecosystems, destroying their habitats and contaminating their bodies. Along with animals, bacteria are present through all ecosystems, breaking down organic materials such as crude oil as a food source (Atlas & Hazen 2011). These bacteria perform an extremely important task of bioremediation, the breaking down of organic waste by biological organisms. Bioremediation happens consistently in the natural environment, removing small amounts of naturally occurring waste. Some amounts of crude oil are broken down by these decomposing bacteria, tackling the lightest, or shallower, forms of organic matter. This allows for shorter residence of crude oil within ecosystems and reduced opportunities for environmental harm. While these bacteria are present in many different ecosystems it is unknown what environment they thrive in.

Background

Bioremediation tactics have been studied for years, establishing the naturally occurring rate of degradation for contaminated soil samples. Crude oil degradation in sediment samples collected off the Chennai coast, in the Gulf of Mexico, after the Exxon Valdez spill were measured for concentration of Polycyclic Aromatic Hydrocarbons (PAHs) and Total Petroleum Hydrocarbons (TPH) to quantify the degradation over 3 years. The study found crude oil is highly mobile through the sediment systems with complex hydrocarbons deeper within the soil samples, while simple hydrocarbons were closer to the surface. The difference in soil grain size allowed for increased diffusion of crude oil across the system, loose grain systems like coarse sands allow greater movement of crude oil while smaller sediments such as clay allow very little movement of crude oil through the system (Tamizhdurai et al. 2022). This movement is associated with the bacteria's ability to access the oil for degradation.

While bacteria are naturally occurring within soil systems, there are similar strains of biodegrading bacteria that exist within the open ocean. These strains were further studied to support their efficiency. Da Rocha et al. (2023) builds off the current understanding of bioremediatory bacteria by identifying *L. vannamei* as a notable bacteria capable of degrading several PAHs. *L. vannamei* was accumulated through biological waste from shrimp allowing for significant concentration of laccase, the enzyme responsible for PAHs breakdown. The study supports that laccase derived from *L. vannamei* can significantly and effectively contribute to the

breakdown of crude oil in oceanic environments. *L. vannamei* was gathered from shrimp tails, purifying the strain and monitoring enzyme activity. The laccase was then added to the collected sediment samples from Cupe Beach, Brazil and PAH concentration was monitored. The study concluded that 50% of detectable hydrocarbons were degraded by the laccase enzyme over 48 hours.

Panchal et al. (2018) provided a unique understanding of the oil emulsification process used to break down oil plumes within ocean environments. The study provides support that oil degrading bacteria are able to attach to oil particles at a higher rate when halloysite (clay nanotubes) are present; the clay particles created a water-oil emulsion that allows for greater mixing of bacteria with oil particles. This study collected naturally occurring deposits of halloysite which were added to a contaminated soil sample along with *A. borkumensis*. Bacteria activity in the halloysite sample was compared to non-halloysite bacteria activity to determine which had the most oil degradation. The study found that *A. borkumensis* most effectively degraded crude oil with Halloysite particles present, the more efficient emulsification made it easier for *A. borkumensis* particles to break down the oil.

While bacteria perform bioremediation, they usually live inside the intestines of other organisms to allow for better mobility. The easiest carriers for this bacterium for this study are nematodes; *A. borkumensis* can survive in nematode intestines for 22 days living on pure crude oil, providing long-lasting bioremediation. The worms provide a viable mode of transportation for bacteria through the sediment systems, allowing for greater access to deeper oils (Shaikhulova et al. 2021). While this experiment will refer to the actions of the bacteria, it is understood that the bacteria is in the nematodes' intestines.

Need

The effectiveness of *A. borkumensis* bacteria has been studied in singular sediment systems and their efficiency has been supported (Tamizhdurai et al. 2022). While studies support that bioremediation can be used to degrade crude oil, the majority have been observational studies that monitor the decomposition of oil from large oil spills. This study will contribute to more specific details about conditions that bacteria prefer and compare efficiency across sediment systems. While understanding the efficiency in medium sand systems is important, oil spills occur across all different kinds of systems supporting the need for multisystem research. This experiment will provide further insight into what oil spill prevention methods to use based on the location of the spill. While bioremediation may be most effective in clay sediments, hair mats could be most effective in the open ocean. Having this data would save money on ineffective clean up methods and save ecosystems from further damage. Reducing the spread of oil and protecting ecosystems will help to save the biodiversity of animals and protect recreational beaches for humans, allowing for greater ecosystem prosperity. Sediments in oil spill environments will reduce oil diffusion and movements while also providing beneficial food for

bacteria. Because of this, sediment environments provide helpful clay particles that increase bioremediation productivity and efficiency.

Hypothesis

Clay sediments will reduce the movement of crude oil, allowing for bacterial breakdown at faster rates. Because of the small pore spaces between the clay particles the oil is unable to disperse throughout the system, allowing for bacteria to enter the small pore spaces and decompose the oil. Open ocean systems on the other hand allow for great dispersion and movement of crude oil, reducing availability to the bacteria. The null hypothesis states that sediment systems do not influence the breaking down of crude oil, supporting a lack of relationship between the variables.

Objective

This study will address oil clean up methods on sediment systems. Because sediments influence the movement of oil, a cleanup must be approached differently based on the sediments that the spills happen on. This study will identify whether clay, medium sand, coarse sand or open ocean are ideal conditions for bioremediation using *A. borkumensis*. If smaller sediments, such as clay, hold on to the crude oil at larger rates, then the bacteria will be better able to break it down. If our hypothesis is true, the clay samples will have the lowest rates of crude oil, and the open ocean will have the highest. Supporting that clay sediments are the optimal environment for bioremediation. Coastal sediments were gathered from nearby, cleaned and separated into petri dishes before oil and bacteria were applied. The percent PAH remaining was collected and the results showed a higher rate of bioremediation in clay sediments, supporting the hypothesis.

Methods

Sediments were gathered from 2-4 feet below the surface at 3 sites, simply randomly selected, along coastal regions to ensure that at least 5 grams of clay, coarse and medium sands can be found. These samples were then used to determine the efficiency of bioremediation across different sediment systems versus open ocean

Sediments were collected along the Rappahannock Riverbank; the sediments were placed through a grain sifter separating each particle; clays (<0.063 mm), medium sands (0.25- 0.5 mm), and coarse sands (1- 2 mm). These sediment sizes were removed from the rest of the collected sample and cleaned thoroughly with Milli-Q Ultra-Pure water. The sediments were placed into containers filled with Milli-Q water multiple times until the water ran clear, and the sediments were visibly clean. This was repeated for each sediment size, then the sediments were

dried in an oven for 24 hours at 50 C. While sediments were drying, the marine broth solution was combined in accordance with manufacturer's instructions. *C. elegans* nematodes were obtained from a manufacturer and fed with *e. coli* before being introduced to sediment samples and stored in marine broth.

0.3g of each size sediment were added to a glass petri dish along with 0.15g of Milli-Q water to moisten the sediments, repeated 12 times per sediment size. As well as this, 12 petri dishes were filled with marine broth and no sediments. Before being added to each petri dish, the nematodes were fed *A. borkumensis*. The nematodes were then washed with Milli-Q water and allowed to air dry before being added to each sediment sample. Approximately 15 worms were introduced to each petri dish randomly.

300 μ L of crude oil was added to each petri dish and placed in a 25 C dark room for 22 days, which is the average lifespan of nematodes living off crude oil (Shaikhulova et al 2021). After the 22-day period, the worms were removed by hand, and Dichloromethane (DCM) was added to remove the remaining hydrocarbons. As DCM is added to the solution, the sediments and water samples were emulsified, collecting the remaining hydrocarbon solution into the DCM mixture. The DCM mixture was then placed into a Gas Chromatography-Mass Spectrometry Machine (GC-MS), used to calculate the remaining percentage of crude oil in the DCM solution extracted from the sediment samples.

The Shapiro-Wilks test and Levene test were run to determine normality and equal variance, the null hypotheses (rejected when <0.05) were that there is a normal distribution or equal variance of data. The dataset passed both assumptions, and was then analyzed using a one-way ANOVA test, comparing the mean remaining PAH percentage across all sediment systems. The null hypothesis for this test is that all of the means are the same, supporting no correlation of data. The null hypothesis is rejected with a p-value less than 0.05 and accepted with one greater. Description statistics were collected; the mean and standard error of the mean were graphed for each sediment in Figure 1.

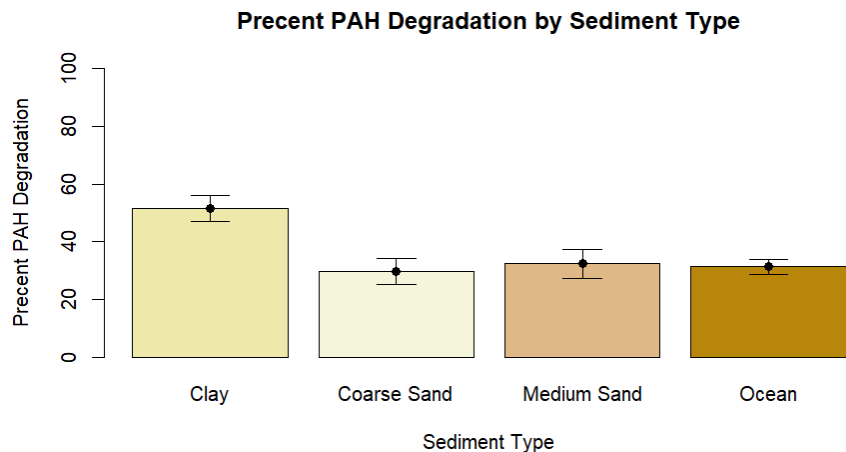


Figure 1. Percent PAH Degradation by Sediment Type

The mean percent PAH degradation of each sediment type was collected and graphed along with the standard error of the mean as error bars. The Tukey test supported that the mean of clay sediments ($58.6 \pm 18\%$) was statistically different than all other sediment types, coarse sand ($33 \pm 14\%$), medium sand ($40 \pm 15\%$), and open ocean ($28 \pm 12\%$).

Results

Figure 1 shows the graphed means and standard error of the mean for sediment types, clay ($58.6 \pm 18\%$ PAH reduction), coarse sand ($33 \pm 14\%$ PAH reduction), medium sand ($40 \pm 15\%$ PAH reduction), open ocean ($28 \pm 12\%$ PAH reduction), seen in Table 1. The Shapiro-Wilk test was run across all datasets to support the assumption of normal distribution with a sample size of 12. The clay samples, the coarse sand, medium sand and ocean samples all passed the assumption of normal distribution with p-values greater than 0.05, according to Table 2, failing to reject the null hypothesis. The Levene test was run, providing the dataset with a p-value of 0.454 which is greater than 0.05, failing to reject the null hypothesis and supporting equal distribution, as seen in Table 3. The ANOVA was run providing an overall p-value of 0.00216, which is less than 0.05, rejecting the null hypothesis and supporting a difference in means of the dataset, see Table 3. A Tukey test was run for further details, presented in Table 4 as well as a graphical representation in Figure 2. The Tukey test expanded on the ANOVA showing which variables were significantly different. All other sediments compared to clay showed a significant difference in means, supporting that clay is the best environment for bioremediation.

Table 1. Means and Standard Error of Mean for PAH Reduction

Descriptive Statistics	Mean (% PAH reduction)	Standard Error of Mean (% PAH reduction)	Sample Size (N)
Clay	58.6	18	12
Coarse Sand	33	14	12
Medium Sand	40	15	12
Ocean	28	12	12

Table 2. Shapiro-Wilks Test Results

Shapiro Wilks	P-value	Test Statistic (W)	Statistical Conclusion (of the null hypothesis)

Clay	0.134	0.894	Fail to reject
Coarse Sand	0.977	0.979	Fail to reject
Medium Sand	0.829	0.963	Fail to reject
Ocean	0.401	0.932	Fail to reject

Table 3. Levene Test and ANOVA Results

Test	P-value	F value	DF	Statistical Conclusion (of the null hypothesis)
Levene	0.454	0.891	3	Fail to reject
ANOVA	0.00216	5.713	3	Reject

Table 4. Tukey Test Results

Tukey	P-value	Statistical Conclusion (of the null hypothesis)
Coarse Sand-Clay	0.004	Reject
Medium Sand-Clay	0.015	Reject
Ocean-Clay	0.009	Reject
Medium Sand-Coarse Sand	0.969	Fail to reject
Ocean-Coarse Sand	0.992	Fail to reject
Ocean-Medium Sand	0.998	Fail to reject

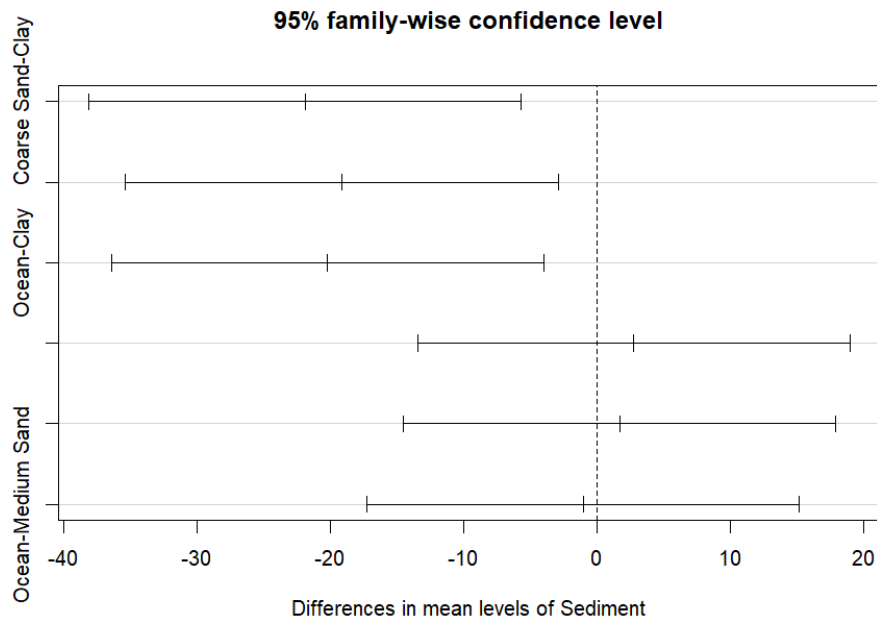


Figure 2. Tukey Test Confidence Interval

The means of each sediment type were compared using a Tukey test, those results were collected and graphed above. The bars of each comparison that intersect the 0 line are not statistically different whereas the lines not touching the 0 line are statistically significant. The means of all sediment systems compared to clay are statistically different, as seen in Table 4 with p-values less than 0.05.

Discussion

The ANOVA accepted the hypothesis, supporting that clay sediments are the best environment for bioremediation. While oil spills can occur in and affect all sediment systems, this study finds that bioremediation is most effective in clay sediments due to the fine grains ability to reduce oil movement, while other sediment sizes had larger pores allowing for oil mobility. This study supports the idea that ocean systems are not under the right conditions to use bioremediation, emphasizing the need for other methods of removing crude. Some innovative solutions such as using discarded human hair as absorption mats could be better used in ocean environments. These mats could be applied directly to the surface, whereas in sand and clay environments these mats would be ineffective to absorb oil from sediments (Erpinar et al. 2024). Other methods such as fibers derived from the jute plant can be used as natural adsorbents for crude oil spills (Srishti et al 2023). While a lot of different remediation options exist, they are all presented as a one-size-fits-all approach, which can be problematic.

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