The Isolation, Enumeration, and Comparison of Aerobic Hydrocarbon Degrading Bacteria in the St. Johns River

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Abstract

Recent events, such as the Deepwater Horizon oil spill, have called attention to the need for the safe and effective removal of oil contamination from the environment. Determining the natural process of hydrocarbon bioremediation within specific habitats is crucial our ability to develop alternative methods of bioremediation. This investigation isolated and characterized the aerobic hydrocarbon degrading bacterial community in the St. Johns River of North Florida. It was hypothesized that the further downstream the site of the sample was taken, the greater the variety of oil degrading bacteria.

Soil samples were taken from potential oil contaminated areas along the St. Johns River and inoculated into media with hydrocarbons as the sole source of carbon in order to isolate and identify strains of hydrocarbon degrading bacteria. Bacteria isolated were then compared for their ability to degrade the hydrocarbons which contaminate the river.

Of the eight unique strains identified, six were isolated from the furthest downstream location. The *Acinetobacter* and *Pseudomonas* species isolated form downstream locations were found to have the best hydrocarbon degrading ability.

The soil samples taken from downstream locations within the river had not only a greater variety of hydrocarbon degrading bacteria but also ability to degrade hydrocarbons than upstream samples.

Introduction

Due to the increased need for oil products worldwide and extensive drilling by companies, oil spills continue to be an ever present threat to the environment. Oil companies have begun to drill deeper and, although more safety precautions are now in place, when accidents happen, the results can be catastrophic. The first major oil spill in U.S. history, involving the Exxon Valdez, captured the public's attention in 1989 when the oil tanker ran aground releasing over forty million liters of oil into the Prince William Sound in Alaska (Hsieh 2010). The Deepwater Horizon spill which happened just two years ago has once again exposed the need for a more effective way to deal with these disasters. Many researchers have been experimenting with bioremediation techniques in the Gulf of Mexico (Kostka 2011). However other researchers have experimented in smaller bodies of water such as rivers or lakes (Hilyard *et al.* 2008). Bioremediation is the process of cleaning up the environment using microorganisms and is a very promising option for removing the oil released by spills. Even though research in this field has shown potential in laboratory settings, adjustments are necessary for large-scale application.

The majority of oil pollution that is typically found in a river from boat and factories run-off is often only dealt with on a visual level, which is sometimes not enough to prevent ecological damage. To be able to maintain the environment at suitable conditions, an understanding of the microbial communities and their impact on the environment is necessary. To deal directly with

oil pollution, people must identify the bacteria aiding in oil disposal. This will allow them to preserve a healthy ecosystem and prepare for oil related accidents. Very little knowledge pertaining to the microbial community of the Lower St. Johns River Basin is available. Stationed on the St. Johns River are many potential sources of contamination, such as power plants, naval stations, and ship yards. Due to these potential sources of pollution it is very important to identify and monitor the microbial community, specifically the hydrocarbon degrading bacterial community.

This experiment hypothesizes that a higher variety of hydrocarbon degrading bacteria will be found downstream as compared to upstream, since the pollution would flow downstream and accumulate at a point.

Material and Methods

For this experiment, a minimal solid and liquid oil enriched media were prepared to select for hydrocarbon degrading bacteria. For the liquid media, a stock solution of distilled water was combined with 20 g sodium chloride (NaCl), 0.250 g of ammonium chloride (NH4Cl), 0.50 g of potassium chloride (KCl), 0.20g of potassium phosphate (KH2PO4), 3.0 g of magnesium chloride (MgCl2), and 0.150g of calcium chloride (CaCl2) was prepared and then was stirred until dissolved completely in the solution. The volume was adjusted to 1000 ml or 1 liter. This solution was sterilized by autoclaving it for 30 minutes.

After the solution was tempered at room temperature, or cooled enough to handle, 10 ml oil solution was added to the salt/ distilled water solution to create a 1% oil solution. The tops of the flask were flamed before adding solution to prevent contamination. The solution was distributed into sterile containers with a sterile pipette for use for later and was labeled with date made to create stock supply of oil enriched liquid culture media.

After the liquid media was prepared, a solid media was prepared by combining the salt/distilled water solution with 15 grams of agar powder. This solution was sterilized by autoclaving it for 30 minutes.

The solution was stirred until evenly mixed. After the solution was tempered at room temperature, or cooled enough to handle, 10 ml oil solution was added to the salt/ distilled water solution to create a 1% solution. Fifteen mL of solution was added to individual sterile Petri dishes and was allowed to solidify at room temperature. The top of the flask containing the solution was flamed before adding solution to the Petri dishes to prevent contamination. Petri dishes were stored in sterile plastic bag and marked with date poured to create plates with oil enriched solid media.

Five cm core samples of soil from the banks of the Lower St. Johns River Basin at potential oil contaminated sites were collected using sterile polypropylene tubes and sterile gloves to extract the soil. These samples were collected at public access areas around the chosen sites: Naval Air

Station Jacksonville, downtown shipping yard, and the Mayport Naval Station. Soil samples were placed in sealable sterile plastic bags. The soil samples were transported in a cooler at 4^{o} C directly to a laboratory setting.

Using sterile gloves, each soil sample was added to a sterile 250 ml flask containing 125 ml of sterile hydrocarbon enriched liquid media prepared earlier and was stored at room temperature on a shaker table at 200 RPM for eight days. The tops of the flasks were sealed with a sterile foam stopper and then covered with sterile aluminum foil, to allow restricted air flow. After the eight day period, each of the solutions were inoculated onto hydrocarbon enriched solid media prepared earlier using sterile gloves to hold a sterile disposable inoculating loop and stored at room temperature for two weeks. The bacterial growth on the plates was sampled by touching a sterile disposable inoculating loop to the colonies of different strains of bacteria and inoculated onto another plate to isolate bacteria using a streaking method. This was repeated until pure strains were obtained. Isolated strains were sent off for identification via DNA extraction and 5S ribosomal unit sequencing.

The results from the gene sequences were recorded and analyzed. The sequences were compared to known sequences of organisms using a nucleotide database, Nucleotide Blast. If the sample was found to have a 99% or greater similarity with a known organism's gene sequence, it could be classified as that organism.

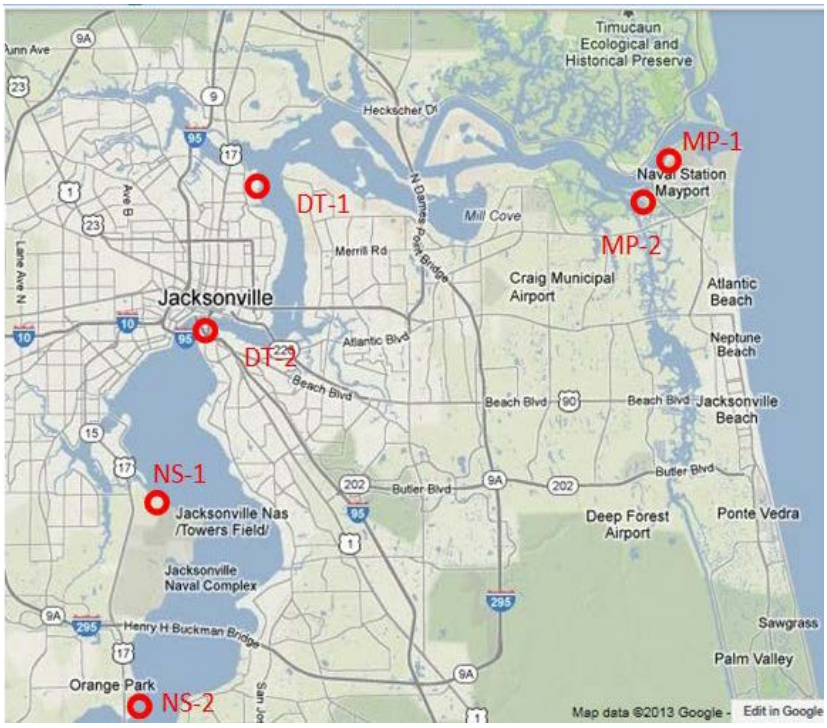
Identified isolated strains of bacteria were inoculated using a sterile inoculating loop into a 250 ml flask with 100 ml of sterile oil enriched liquid media containing 10 ml of sterile oil solution, a combination of used motor oil and diesel fuel. The tops of the flasks were flamed when solution was added and then sealed with a sterile foam stopper and covered with sterile aluminum. The solutions were stored at room temperature for two weeks on a shaker table at 200 RPM.

The oil solution from the flasks was siphoned off into 10 ml test tubes and centrifuged to separate oil from the bacterial biomass. Then the degradation between different strains of isolated bacteria was analyzed by measuring the height of oil remaining and calculating its volume.

Results

	Locations where isolates were
Pseudomonas putida	MP-1
Acinetobacter venetianus	DT-1, DT-2, MP-2
Salibacter luridus	MP-1
Mangroveibacter sp.	NS-1
Pseudomonas sp.	MP-1
Gallaecimonas pentaromativorans	DT-2
Rhizobium sp.	MP-1, DT-1
Salinicola sp.	MP-2

Site where samples were taken

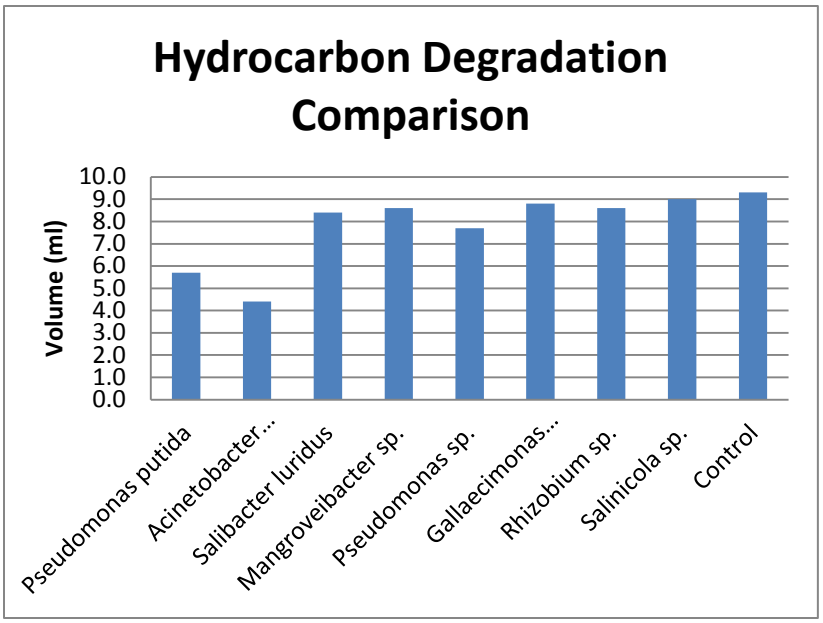


The bacteria identified in this experiment were *Pseudomonas putida, Salibacter luridus, Acinetobacter venetianus, Mangroveibacter* sp., *Pseudomonas* sp., *Gallaecimonas pentaromativorans, Rhizobium* sp., *and Salinicola* sp.

Height of oil mixture in vial (mm)

Pseudomonas putida	43.0
Acinetobacter venetianus	33.0
Salibacter luridus	63.0
Mangroveibacter sp.	65.0
Pseudomonas sp.	58.0
Gallaecimonas pentaromativorans	66.0
Rhizobium sp.	65.0
Salinicola sp.	68.0
Control	70.0

	Volume of oil remaining (ml)
Pseudomonas putida	5.7
Acinetobacter venetianus	4.4
Salibacter luridus	8.4
Mangroveibacter sp.	8.6
Pseudomonas sp.	7.7
Gallaecimonas pentaromativorans	8.8
Rhizobium sp.	8.6
Salinicola sp.	9.0
Control	9.3



The isolate that exhibited the greatest ability to degrade hydrocarbons used in this experiment, was *A. venetianus*, which was able to degrade 53% of the hydrocarbon present as compared to the control and was 20% more efficient than the next best hydrocarbon degrading, *P. putida*, which degraded 39% of the hydrocrbons. Another relatively efficient degrader was *Pseudomonas* sp., which degraded 17% of the hydrocarbons. Other significant degraders were *S. luridus*, *Mangroveibacter* sp., and *Rhizobium* sp., which degraded 10%, 8%, and 8% respectively. The least efficient hydrocarbon degraders tested were *G. pentaromativorans* and *Salinicola* sp, and did not degrade a statically significant amount of oil compared to the control.

Discussion

Since the greatest number of different strains of bacteria able to degrade oil was found at the farthest downstream area, this suggest that the pollution found in the river was greatest at this

point and infers greatest acculation of pollution. The bacteria living there have adapted to be able to survive and even thrive in the presence of oil. Many of the bacteria isolated in this experiment are known oil degrader. The bacteria found to degrade the greatest amount of hydrocarbons in this experiment was *Acinetobacter venetianus*. This bacteria, *A. venetianus*, has been characterized by other researchers to be able to degrade diesel fuel, which would have been a major pollutant found in the St. Johns River because of boating traffic. *P. putida*, in fact, was the first patented hydrocarbon degrading bacteria developed by scientist. The presence of these oil-degraders suggest that a source of pollution has collected or is ever present in the environment, but also means that remediation of the pollution put forth by humans is currently occuring and is relatively effective. The bacterial strains were grown in a minimal media and were still able to degrade a significant amount of the oil. Further testing is necessary for a comparison between minimal conditions and biostimulation.

Due to the time constraints of this project, the number of testing repetitions was limited. Also the oil mixture of diesel fuel and motor oil used in this experiment may have limited the variety of bacteria obtained. However the mixture used targeted the major pollutants found in the St. Johns River and tried to provide the greatest variety of hydrocarbon possible. In future experimentation a wider range of hydrocarbons should be introduced or a specific hydrocarbon should be tested for.

Conclusion

The data collected supported the hypothesis, concluding there were a greater variety of hydrocarbon degrading bacterial strains identified downstream as compared to upstream with in the Lower St. Johns River Basin. Out of the eight strains isolated and identified by genetic sequencing, six came from the farthest downstream site, three came from the mid-river site, and one came from the farthest upstream site, with two species coming from both the farthest up stream site and mid-river site. The most efficient hydrocarbon degrader found was from the farthest downstream site and mid-river area. The second and the third were also found at the farthest downstream site.

Due to the increased need for oil products worldwide and extensive drilling by companies, oil spills continue to be an ever present threat to the environment. Not only do these spills damage the surrounding ecosystems, but also many industries around the spills. To keep up with the ever increasing demand for crude oil, companies have begun to drill into deeper water, and although there are many safety precautions in effect, when something goes awry the results can be disastrous. Physical and chemical removal of the oil is often labor intensive and costly, while being harmful to the environment. Bioremediation offers promising results, since it is both fairly efficient and inexpensive, while being environmentally friendly. Since one type of bacteria generally degrades a specific part of crude oil, it has, in some cases, been used in the oil refinery process. Basic understanding of the natural occurring bacteria that are able to degrade hydrocarbons is a necessary to be able to fully exploit all possibilities of bioremediation.

Research in the field of bioremediation still is in its infancy and still has many opportunities to improve its efficiency. A major problem for bioremediation is the dispersal of oil to the bacteria. The larger the surface area of the oil, the more efficiently the bacteria and other microbes can degrade it. To solve this problem many researchers have been looking into the possibility of biosurfactants, produced naturally by some bacteria, which will increase the overall contact area between the oil and the microbe. Another interesting field of research is using bioluminescence genes as biosensors to monitor petroleum-based environmental pollutants. Additionally, researchers are investigation the application of using horizontal gene transfer to make a certain type of bacteria more efficient at degrading a larger array of components in crude oil. When I continue this research, I would be interested in using horizontal gene transfer to try to develop a more effective bacterium able to purify factory runoff.

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