

## Final Report

### ***Exploring the Diversity and Biopharmaceutical Potential of Uncultivable Bacteria from Lake Michigan Sediments***

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**Abstract:**

We performed five large isolation experiments using a simulated natural environment during the grant period in an attempt to obtain previously unculturable microorganisms from Lake Michigan. The initial experiment had to be halted due to the presence of *E. Coli*, which outcompeted all other bacteria. We unsuccessfully tried to avoid *E. Coli* by changing our sampling location and/or methods. Both times the presence of *E. Coli* interfered with the experiment. To overcome the *E. Coli* overgrowth problem, we used a heat treatment protocol, which removed all non-spore forming bacteria. This was successful and no *E. Coli* overgrowth occurred during our final two experiments. Due to the slow growth rate of the remaining microorganisms we had to increase the time spent in our simulated natural environment. This increased experimental time was not compatible with our membranes and resulted in compromised integrity of the membranes sealing our diffusion chambers during our fourth experiment in fall of 2007. A final experiment using a

protocol to overcome this problem was initiated in summer of 2008 and is still ongoing at the time of this report.

### **Introduction:**

The overall objective of this research was to explore microbial diversity in Lake Michigan for its biomedical and biotechnological potentials. We are focusing on a unique source of previously unculturable aquatic microorganisms. The long-term goal of this research is to further our understanding of the diversity as well as chemistry and biochemistry of these aquatic microorganisms in Lake Michigan. The specific aims of this project were:

1. To isolate and grow previously unculturable aquatic microorganisms.
2. To evaluate extracts for anticancer and anti-tuberculosis activities.
3. To evaluate active extracts for the presence of known active metabolites (dereplication).

### **Narrative Report:**

#### *Background:*

The majority of microorganisms from the environment resist cultivation in the laboratory. These “uncultivables” represent 99-99.99% of all microorganisms in nature. This phenomenon has been recognized as great challenge, since genetic evidence suggests that diverse uncultivated microbial taxa dominate most natural ecosystems.

Recently the group of Dr S. Epstein at Northeastern University developed a general method to grow “uncultivable” bacteria by placing them in a simulated natural environment in a diffusion chamber (Kaeberlein *et al*, Science, 2002, 296, 1127-1129). The concept is

based on a simple observation that the microorganisms appear “uncultivable” only *in vitro* (=on a Petri dish), whereas *in situ* they seem to be growing (at least periodically). It follows that the natural environment provides microorganisms with the essential growth conditions in an appropriate combination. To achieve these conditions, an environmental sample is inoculated in a growth chamber and separated from the environment by semi permeable membranes. These membranes physically prevent cell migration and thus maintain purity of the inner space, yet enable full chemical contact between the inner space and the outside environment. The inoculated, sealed chambers are placed on the surface of sediment collected from the tidal flat and kept in an aquarium. Thus, microorganisms inside the device were exposed to the complete suite of the outside environmental conditions and components. Using this method they observed that approximately 300-fold more microorganisms produced sustainable growth in the chambers than in standard Petri dishes. The methodology was developed to obtain “uncultivable” marine microorganism, and during this project we aimed to adopt this methodology for fresh water microorganism from Lake Michigan.

#### *Methods:*

We installed two 39 gallon fiberglass tanks with water pumps for water circulation in our laboratory to be able to establish a simulated natural environment for bacterial growth. Sediment and water samples from various Lake Michigan habitats were collected, transported to our laboratory, and placed in the tanks. We performed a total of five sediment and water collections during the grant period. The initial two collections were obtained from Illinois State Beach in June and August of 2006. Two additional collections were performed in late June and in early September of 2007 at locations at Warren Dunes State Park, MI. One final collection was done in early July of 2008 also at Warren Dunes

State Park, MI. Each collection consisted of two autoclave trays with surface sediment and 100L of Lake Michigan water (figure 1A and 1B). Upon arrival in the laboratory, the water was added to the fiberglass tank and the sediment trays were placed in the water (figure 1C). The water was circulated using a water pump. This simulated natural environment was left to equilibrate for 24-48 hours before samples were added (figure 1D).

We collected four samples of the surface layer of the sediment by scraping the top 2-3 mm of the sand into sterile tubes at each collection site. These samples were individually processed for the microbial isolation. The microorganisms were detached from the sand grains in the laboratory by vortexing in autoclaved Lake Michigan water. The sediment was allowed to settle for 5 minutes and the supernatant was observed under the microscope. An aliquot of the supernatant (100-300  $\mu$ l) was removed and mixed with 0.7% agar made with sterile lake water and poured into a diffusion chamber followed by sealing of the chamber. In addition, two ten fold dilutions were made of each supernatant and mixed with 0.7% agar made with sterile lake water and poured into a diffusion chamber. Each sample was prepared as a duplicate for a total of 6 diffusion chambers per sediment sample. The diffusion chamber was formed by a stainless steel washer sandwiched between two polycarbonate membranes (0.03- $\mu$ m pore-size, GE Osmonics Microporous Polycarbonate Membranes). The membranes were glued to the washer using a silicone sealant (GE Silicone Glue II) to form the inner space of the diffusion chamber filled with microorganisms in 0.7% agar. The inoculated chambers were placed on the surface of the sand sediment in the simulated natural environment of the tank (figure 1E).

The chambers were removed after about two weeks, the top membrane detached, and the samples were observed under the microscope (figure 1F). Observable colonies of microorganisms were removed and serial diluted with sterile lake water. The dilutions were

mixed with 0.7% agar and poured into new chambers. The chambers were again placed in the aquarium for two weeks. In cases where no colonies could be observed, portions of the chamber content was removed, mixed with sterile lake water followed by 0.7% agar and poured into new chambers. These chambers were also placed in the aquarium for two weeks.



Figure 1 Set up of diffusion chambers and simulated natural environment. (A) Field collection of sediment. (B) Field collection of water using 25L carboys. (C and D) Simulated natural environment set up in laboratory using collected Lake Michigan sediment and water. (E) Diffusion chambers incubated on the surface of the sediment. (F) Diffusion chamber being opened for observation after incubation in the simulated natural environment.

### *Results:*

The initial collection from Illinois State Beach in June of 2006 was processed as described above. The diffusion chambers showed the presence of large amounts of *E. Coli* already at the first passage and the experiment was discarded. The *E. Coli* contamination was initially thought to be due to the sediment collection site (beach wave zone). Thus the second collection from August 2006 was done at 3-4 ft depth of water. This collection also had to be halted due to the large amounts of *E. Coli* found.

To minimize the *E. Coli* contamination problem, we decided to try to avoid runoff areas from the more populated areas around Chicago for our sampling. We thus moved our primary collection site to Warren Dunes State Park, Michigan. The initial observation of the June 2007 collection from this site indicated the presence of *E. Coli* in the samples. The levels were, however, much lower than found in previous collections from Illinois Beach State Park and we decided to continue the experiment. A portion of the chamber content was removed and serial diluted with sterile lake water. The dilutions were mixed with 0.7% agar and poured into new chambers. The chambers were again placed in the aquarium for two weeks. Again, the chambers were removed and observed under the microscope. At this point *E. Coli* was found in all samples and had out competed all other bacteria in our diffusion chambers. The experiment was halted. The aquaria were cleaned and the water and sand discarded.

To overcome the *E. Coli* overgrowth problem, we decided to pre-treat our samples using a heat treatment protocol to remove non-spore forming bacteria (including *E. Coli*) prior to inoculation in the chambers. This new method represents a necessary step to avoid the *E. Coli* contamination, but the heat treatment also removed all non spore-forming bacteria and changed the species diversity we could obtain from our method.

Nevertheless, we employed this method for our second sediments and water collection from early September 2007. We followed the protocol outline above with the modification that the vortexed sample was heated at 60°C under constant shaking for 5 min prior to dilution and mixing with the agar. The content of the chambers were observed after 14 days and no *E. Coli* overgrowth was observed. The number of observed microbial colonies was however very low and the colonies were very small. Still portions of the agar was removed, diluted, and poured into new chambers. These chambers were placed in the aquarium for three weeks to increase the growth and colony size of the bacteria. After three weeks the number and size of observed microbial colonies was still very small. Again, the agar was removed, diluted, poured into new chambers, and left for six week in the aquarium. After six weeks the integrity of membranes was compromised with holes and all the chambers were contaminated. This represented a major set back, but we did establish that our heat treatment worked to prevent *E. Coli* contamination. Unfortunately, the growth rate of the surviving bacteria is very slow and additional modifications had to be incorporated in our protocols.

In addition to the inoculation into diffusion chambers, a portion of each sediment supernatant sample was inoculated on a regular Petri dish containing 1.5% agar in autoclaved Lake Michigan water and stored at room temperature for 2 weeks to access number of cultivable strains in the samples. Several colony types were observed from each sample. However, the observed colony number decreased considerably after our heat treatment as was expected.

We performed one sediment and water collection in July of 2008 at Warren Dunes State Park, Michigan. This experiment is still ongoing, but our initial data looks encouraging. We observed no *E. Coli* contamination after two passages in the diffusion

chambers and several bacterial micro-colonies were observed. We are now working under the assumption that we have to perform a chamber exchange every three weeks. We will continue this experiment for another two to three rounds to try to increase the cell numbers of the observed micro-colonies.

*Conclusions/recommendations:*

The method of obtaining previously unculturable microorganisms using diffusion chambers and a simulated natural environment was difficult to adapt to Lake Michigan from the marine environment for several reasons. The major reason was the presence of large numbers of *E. Coli* bacteria in all our collections. The *E. Coli* outcompeted and overgrew all other bacteria in our experiments. This problem was never observed while working with marine samples, since, even when present from run off, *E. Coli* does not grow to any observable extent in saltwater. We were forced to adjust our protocols to eliminate *E. Coli*. Our method of choice (heating at 60°C for 5 min) removed all non spore-forming bacteria from our sample. While this method removed *E. Coli*, it also significantly altered the microbial species diversity in our samples and limited the range of organism we can obtain using diffusion chambers. The heat treatment lowered the numbers as well as size of the microbial colonies observed, which forced us to increase the time of the experiments. This in turn was not compatible with our experimental set up. Our lack of success to date to isolate and grow previously unculturable aquatic microorganisms has been very frustrating. It also prohibited us from making progress to accomplish our subsequent aims (biological evaluation and chemical dereplication). This general approach is being successfully used in our other project aimed at investigation of cultured cyanobacteria. We are hopeful that we have addressed the issues regarding the growth of

previously unculturable aquatic microorganisms and that our ongoing experiment will be more successful.

Recommendations for future experiments would include addressing the following issues:

1. How to overcome the *E. Coli* problem without significantly alter the microbial make up the samples?

This may be done by pre-treating the sample with specific antibiotics against gram-negative bacteria or by a more gentle physical method. It would also be of interest to collect samples from more pristine Lake Michigan areas or from greater depths to potentially avoid the presence of *E. Coli*.

2. How to increase the microbial growth rate in the diffusion chambers?

This could include the addition of nutrients to the diffusion chambers and/or tank water. It would also be of interest to increase the water temperature by adding a water heater to our simulated natural environment. All our experiments were carried out at ambient laboratory temperature (around 22°C). An increase in temperature may speed up the growth.

3. How to adjust the membrane of the diffusion chamber to accommodate the long time needed in the simulated natural environment?

This would include the exploration of membranes made of other materials than the polycarbonate material used in our current membranes.

## **Keywords**

Previously unculturable microorganisms, diffusion chamber, bacterial communication

## **Lay Summary**

The goal of the research was to explore a vast, but untapped resource existing in Lake Michigan - previously unculturable microorganisms. A majority (> 99%) of all microorganisms resist cultivation in the laboratory. We used a method based upon simulated natural environment to attempt to access these organisms. The presence of *E. Coli* in all the samples collected at various locations from Lake Michigan, hindered our initial experiments, since *E. Coli* outcompeted and overgrew all other bacteria. We successfully implemented a heat treatment protocol to remove *E. Coli* from our samples. The growth rate of the previously unculturable microorganisms in our simulated natural environment was very slow and not compatible with our initial set up. We currently have an ongoing experiment to try to circumvent these problems.

## **International Implications**

None during this period

## **Media Coverage**

None during this period

## **Partnerships with other institutions/individuals initiated or continued by your project**

None during this period

## **Publications**

None during this period

## **Undergraduate/Graduate Names and degree**

Shunyan Mo, PhD student, graduation expected in summer 2009

**Related Projects**

None during this period

**Awards and Honors**

Shunyan Mo received the van Doren Scholar award from the UIC College of Pharmacy

**Patents/Licenses**

None during this period