Spring 2014

Investigating the Interactions Between Cyanobacteria and Vibrio parahaemolyticus

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One well-known pathogen that has been the topic of many recent studies is *Vibrio parahaemolyticus,* which causes thousands of foodborne illnesses a year, mostly from the ingestion of raw or undercooked oysters. It has been shown cyanobacteria can act as a long-term reservoir of *Vibrio cholerae*, another pathogenic *Vibrio*, by encasing the cells within mucilaginous sheaths during which Vibrios enter a viable but non-culturable state. In this study we investigated the interaction of *V. parahaemolyticus* with cyanobacteria to determine whether cyanobacteria aid in the longevity and survival of *V. parahaemolyticus*. We found that non-pathogenic *V. parahaemolyticus* strain G445 was able to persist better in the presence of cyanobacteria compared to pathogenic *V. parahaemolyticus* MDOH-04-5M732. G445 cells seem to cluster non-discriminately within the cyanobacterial mats, which may be due to specific interactions with cyanobacteria, yet further investigation is necessary.

1 - Introduction

Over the past few decades the incidences of *Vibrio* related illnesses have steadily increased. At least 30,000 foodborne infections per year are caused by *Vibrio parahaemolyticus* (Scallan *et al.* 2011), typically from ingesting raw or undercooked oysters. Historically, *V. parahaemolyticus* outbreaks have been centralized in the warm waters near the Gulf of Mexico. However, recent incidences of disease have been appearing in regions not previously associated with *Vibrio* outbreaks (Schets *et al*. 2011. This is due in part to changing patterns of human land and resource use along with changes in the environment. The most notable are seasonal temperature extremes, which may represent larger changes in climate (Schets *et al*. 2011).

The emergence of these pathogens in areas in New England and the Pacific Northwest highlight the expanding range of Vibrios and pose a risk for gastroenteritis in consumers of shellfish (Schuster, 2010). Despite the fact that more illnesses are occurring in these areas, only limited research is ongoing on the microbial interactions that influence the emergence of pathogens within the microbial community. Ongoing studies conducted at The University of New Hampshire show that three species of *Vibrio, V. vulnificus, V. parahaemolyticus,* and *V. cholerae,* are present in the Great Bay Estuary. These *Vibrio* are detected seasonally and grow in direct relationships with warmer temperatures, and for some species, lower salinity following a storm (Givens *et al. 2014*). The Great Bay is excellently suited for research due to the wide range of environmental conditions, such as temperature changes and salinity, which occur over the relatively small space. The bay is also home to many natural oyster beds. Oysters can be studied under a variety of abiotic and biotic conditions to see the effects of those conditions on microbes within the oysters, including Vibrios.

Along with warmer temperatures increasing the abundance of *Vibrio*, recent evidence from prior studies suggest a relationship between *Vibrio* and cyanobacteria (Islam *et al*. 2002). Cyanobacteria are photosynthetic organisms, found in almost every terrestrial and aquatic habitat and are well known for their extensive and highly visible blooms that can form in both freshwater and marine environments.

Previous studies have shown that when grown together, cyanobacteria play a role in the persistence of *Vibrio* species; specifically pathogenic *Vibrio cholerae* 0139 and can act as a long-term reservoir for upwards of 15 months. *Vibrio* seemed to be encased within the mucilaginous sheaths of the cyanobacteria, allowing for allowing for a possible symbiotic relationship with the Vibrios. The two organisms may participate in a CO_2 and O_2 exchange (Islam *et al.* 2004). Another study illustrated that the presence of cyanobacterial organic matter significantly influenced *V. cholerae* and *V. vulnificus* growth and abundance, further evidence that the two species may have an association. (Eiler *et al*. 2007).

These findings reveal that cyanobacteria may be assisting in the proliferation of *V. cholerae* in aquatic environments and, as oysters are filter feeders, may be influencing the accumulation of Vibrios in oysters. The major goal of this proposal is to explore the mechanisms of this association between cyanobacteria and *V. parahaemolyticus* in the Great Bay Estuary using both environmental and pathogenic strains. It is hypothesized that cyanobacteria will aid in the growth and proliferation of *V. parahaemolyticus.* The co-occurrence of these species may reflect metabolic syntrophy or simply indiscriminate physical attachment in algal mats.

2 – Methods

In order to test the hypothesis, we designed an experiment testing the persistence of *Vibrio parahaemolyticus* and *Vibrio cholerae* with and without the presence of cyanobacteria using quantitative plating and visualization with confocal microscopy. We tagged both *Vibrio parahaemolyticus* and *Vibrio cholerae* with a green fluorescent protein, which allowed them to be visualized and contrast with the red autofluorescense of purified cyanobacteria.

2.1 – Tagging *V. parahaemolyticus* and *V. cholerae* with GFP

Donor *E.coli* DH5α pVSV102 GFP and helper *E.coli* DH5α pEVS104 cells were streaked onto separate Heart Infusion (HI) Kan50 plates. Recipient *V. parahaemolyticus* strains G445 and MDOH-04-5M732 (referred to as MDOH-04 for the remainder of the paper) were streaked onto an HI plate. The donor cells used for *Vibrio cholerae* were PKV111, which were streaked out onto a Luria Bertani Salt (LBS) Cl 2.5 plate. Recipient *V. cholerae* 2740-80 with a knock-out *toxR* gene (referred to as *V. cholerae* in the remainder of the paper) were streaked out onto an LBS plate.

Five colonies were chosen from the donor and helper growth plates and were inoculated into separate tubes of 3mL HI Kan50 broth while 5 colonies of the recipient were inoculated in 3mL of HI broth. The procedure was completed in the same way for *V. cholerae* with the exception of using LBS broth. The cultures were allowed to grow for 1.5 hours and 3 hours for *V. parahaemolyticus* and *V. cholerae*, respectively. All tubes were grown at 28°C with shaking. After completing the necessary growth time, 500μL of each donor was added to an Eppindorf tube and was spun at maximum speed (13.2 x 1000 rpm) for 2 minutes in a centrifuge (4515D, Eppendorf, Germany). The supernatant was aspirated and 500μL of helper culture was then added to each tube. The tubes were again spun for 2 minutes at max speed. Once that was

completed, the supernatant was removed and 1mL of each recipient was added to individual tubes and spun for 2 minutes at maximum speed. The supernatant was removed and the entire pellet re-suspended in 100µL of broth, either HI or LBS depending on the strain. The entire 100µL was then spot plated onto a HI plate for *V. parahaemolyticus* or an LBS plate for *V. cholerae*. These plates were incubated right side up overnight at 37°C.

The final step was to scrape off the spots that have grown overnight and add them to an Eppindorf tube with 1mL of either HI or LBS. The tube was vortexed for 1 minute to break up the cells. 100µL from each tube was then removed and diluted into 900µL of HI or LBS for *V. parahaemolyticus* and *V. cholerae,* respectively. Finally, 100µL from these dilutions were spread plated onto HI Kan50 plates for *V. parahaemolyticus* and LBS Cl2.5 for *V. cholerae*. Colonies appeared after 1-2 days and were then re-streaked for isolation and then frozen down in glycerol at -80ºC.

2.2 – Cyanobacteria Isolation and Growth

Cyanobacteria mats were collected from a swampy area near Adams Point, NH.

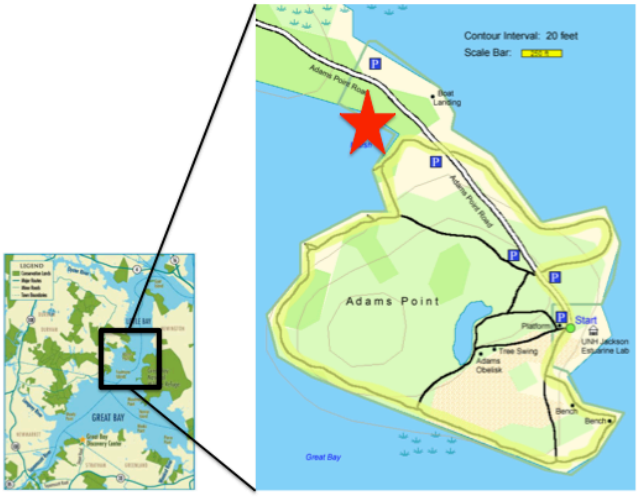

Figure 1: Site of cyanobacterial collection. Cyanobacteria were collected from a swamp (indicated by the red star) located directly by the Great Bay Estuary in Durham, NH.

This swamp is in very close proximity to the Bay water (Figure 1) and it can be assumed the cyanobacteria collected from the swamp are also found in the Bay water; this allowed us to collect from a dense population without the need for filtering and concentrating water samples. There were three different sample sites along the swamp where scoops of muck were removed and bagged. The first site consisted of a very muddy area right off the shore. The second sample was further from shore and contained an approximately even mixture of mud and water, and finally the third sample was chiefly water with very little mud.

Upon observing with a microscope, it was noted the samples were mixed with dirt, nematodes, and other impurities. To isolate the cyanobacteria, 1mL of sample was added into 11.5mL of ASNIII media, a media specifically designed for cyanobacteria enrichment (Supplemental), in sterile flasks with foil lids. There were 6 flasks prepared, two replicates from each of the three different collections sites. The flasks were grown following methodology from Bolhuis *et al*. (2010) with shaking at 120rpm and a light/dark cycle of 14 hours and 10 hours, respectively. The intensity of the light in the growth chamber averaged 70μmol $m^{-2}s^{-1}$. These cultures were grown for two-week intervals, after which the mats that formed were broken up using a sonicator (Frontline Electronics and Machinery, India) to homogenize the samples in solution and kill any impurities still growing. New flasks were prepared with 1mL of homogenized sample and 11.5mL fresh media. This purification process was done every two weeks for a total length of 8 months, after which the cyanobacterial mats were completely green and appeared to be rid of most impurities. After purification, samples were transferred to 25mL of fresh ASNIII media to enhance growth. All other growth conditions remained the same (Bolhuis *et al. 2010).*

2.3 – Determining the Minimal Level of Antibiotics

Because the *Vibrio* strains used in the experiment had to be grown in antibiotic media to induce the production of the fluorescent protein, it was necessary to determine what level of those antibiotics cyanobacteria could persist in. To test all the different levels, which ranged from Kan 10 to Kan 50 for Kanamycin and Cl .5 to Cl 2.5 for Chloramphenicol, a range of three replicate tubes were prepared for all of the different antibiotic concentrations. These included Kan 50, 30, 10, and 2.5, along with Cl 2.5, 1.5, and .5. Each tube contained sterilized GBE water as a solvent into which the antibiotics were added. Each tube was also inoculated with cyanobacteria, roughly the same amount per tube. These were allowed to grow for just over three weeks following the same growth conditions as described above. As the Islam *et al.* (2002) paper indicates, *V. cholerae* enters the VBNC state after 22 days, so a period of three weeks was applicable for determining antibiotic levels.

2.4– Determining Concentrations of *Vibrio parahaemolyticus* & *Vibrio cholerae*

We needed to ensure an equal concentration of each species was used in the growth experiments. Rather than performing a plate count to determine concentration each time, we performed a plate count once and took OD600 readings to convert CFU/mL to CFU/OD so concentration could be determined solely by OD600 readings. First, each of the GFP-tagged strains, MDOH, G445, and *V. cholerae*, were streaked onto antibiotic plates, HI Kan50 for the *V. parahaemolyticus* strains and LBS Cl2.5 for *V. cholerae,* and grown overnight at 28°C. Five colonies from each plate were then used to inoculate 3mL of liquid antibiotic media, with three replicates per strain. The strains were then incubated at 28ºC with shaking until they reached the exponential growth phase, 1.5 hours for *V. parahaemolyticus* and 3 hours for V. *cholerae.*

Once the growth phase was completed, 500μL of growth media, either HI Kan50 or LBS Cl 2.5, was added to cuvettes, with three readings for each replicate. OD600 readings were taken using a spectrophotometer (Biophotometer, Eppendorf, Germany) for each sample, using 500μL of media to blank and then an additional 500μL of sample for each replicate. Following the completion of the OD600 readings, a serial dilution was performed using the remaining media from the original growth tubes. Each was diluted to 10^{-5} into sterilized GBE water. Tubes that were in the range of 10^{-3} to 10^{-5} were spread plated with 100μL of media onto antibiotic plates; the plates were then incubated overnight at 28°C.

After the growth period, all the plates showing between 30-300 colonies were counted and recorded. From this data it was possible to develop a conversion factor for each specific strain, between OD/CFU and CFU/mL, based on the colony counts at specific dilutions. These values could be used each time it was necessary to calculate the number of cells in a particular sample for specific strains, when grown in conditions as described above.

2.5 – Experimental Growth Tubes

In order to investigate the interaction between *V. parahaemolyticus* and cyanobacteria it was necessary to set up specific growth tubes for each strain, following the procedure described by Islam *et. al* (2003) with slight modifications. Each experimental growth tube contained 0.1g of cyanobacteria, 10^6 cells of the strain of interest, and 3mL of sterilized antibiotic GBE water. The strains of interest used were GFP-tagged MDOH-04 and G445 in Kan50 GBE water, and GFP-tagged *V. cholerae* (as a positive control), with Cl2.5 GBE water. Negative control tubes consisted of 10^6 cells of each strain inoculated in 3mL of filtered GBE antibiotic water. Three experimental and three control tubes were prepared for each strain. All tubes were grown in conditions described in section 2.2.

2.6 – Plating Schematic

To determine when *V.parahaemolyticus* and *V.cholerae* would be no longer culturable, a quantitative plating scheme was developed. Starting on Day 0, at approximately the same time each day, dilutions were performed from 10^{-1} to 10^{-5} with 100uL of supernatant from the growth tubes using sterile GBE antibiotic water as a solvent. Dilutions were plated onto HI Kan50 plates or LBS Cl2.5 for *V.parahaemolyticus* and *V.cholerae*, respectively, and grown overnight at 28°C. Colonies were counted the following day to determine the culturable CFU/mL of each strain. Only plates that had between 30 and 300 colonies were counted, otherwise plates were recorded as too numerous to count (TNTC). Once growth ceased on the lowest dilution, that dilution was discontinued the following day. This process was repeated for 9 days, after which there was not enough remaining supernatant the tubes to remove 100μL for dilutions. One the final day, the cyanobacterial mats that remained in the tubes were removed, broken apart by vortexing with about 0.1 gram of glass beads in an microcentrifuge tube with 100uL sterile GBE water, and diluted and plated in the same methodology as described above. All of the plate count data was recorded and analyzed.

2.7 – Statistical Analysis

CFU/mL counts were log transformed and two-tailed T-tests assuming unequal variance were used to determine statistical significance.

2.8– Fluorescent Microscopy

The last essential step of this experiment was to visualize both *Vibrio* and cyanobacteria under a confocal microscope (Zeiss LSM 510, Thornwood, New York) to observe interactions between the two species. Growth tubes with cyanobacteria and G445, and cyanobacteria and *V. cholerae*, were set up and allowed to grow for eight days, to replicate the time used in the plating

schematic, under the same conditions as described above. On day 8, sets of day 0 tubes were also prepared for comparison. Green fluorescent beads (Flouresbrite Microspheres, Warrington, Pennsylvania), at the same concentration of the *V.parahaemolyticus* and *V.cholerae* cells, were inoculated with cyanobacteria for days 0 and 8 to act as a negative control. They were used to determine if particles in the presence of cyanobacteria simply get stuck in the mats. Slides were observed under the confocal at a wavelength of 488nm. All of the images taken are stored in the confocal computer under the Cooper account.

3 – Results

Cyanobacterial mats were able to persist in Kan50 and Cl2.5 for a period of three weeks, judging persistence based on color: if the mats had started to turn light green to yellow, we believe this would indicate cell death. This was not seen in the three-week period. The OD600/mL and CFU/mL conversion were calculated to be 8.39×10^7, 3.63×10^8, and 4.10×10^8 for *V. cholerae*, MDOH-04, and G445, respectively.

With this experimental set up, there are several questions that can be addressed and evaluated using the CFU/mL data collected. In general, trends will be described rather than statistical results unless noted otherwise; at the end of the experiment there were not enough data points to perform statistical analyses (refer to section 2.6).

3.1 – How does each *Vibrio* strain grown with cyanobacteria compare to each *Vibrio* control strain?

G445 grown with cyanobacteria ended at a slightly higher concentration than G455 cells that were grown in the control tubes (Figure 1). MDOH-04 grown with cyanobacteria ended at a significantly higher concentration then MDOH-04 used as a control (Figure 2, $p = 0.008$). Finally, *V. cholerae*, grown with and without cyanobacteria, showed a similar downward growth

trend overall with the control tube showing slightly lower cell concentrations that the experimental tube (Figure 3).

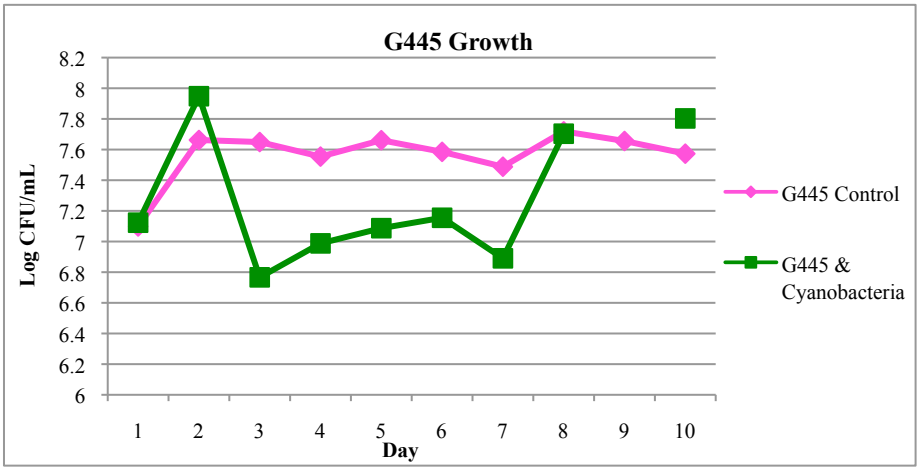

Figure 1 – G445 Grown With Cyanobacteria Compared To The Control. Log-transformed colony count data for the cyanobacteria and *Vibrio* growth tubes, as well as the *Vibrio*-only control tubes from day 1 to day 10. All points represent the average of three replicates, with the exception of day 9 and 10 points, which only represent two of the replicates. There were also no countable colonies for G445 and Cyanobacteria for Day 9; the lowest dilution had too few colonies while the next highest had TNTC.

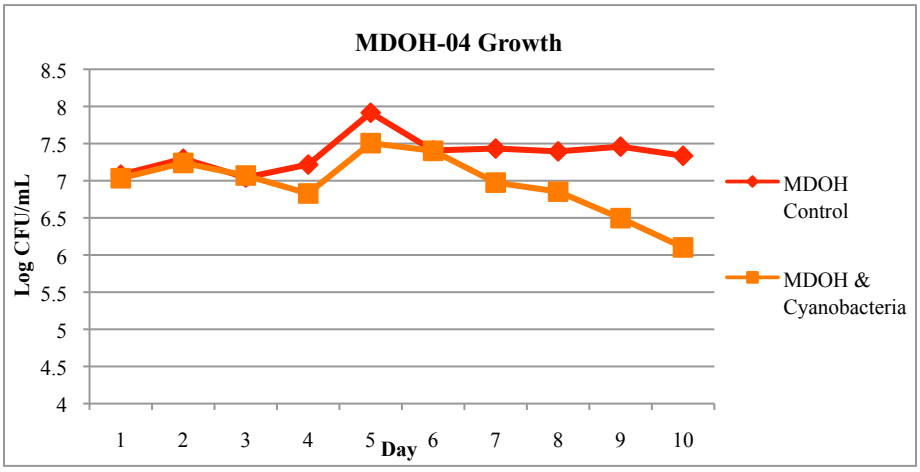

Figure 2 – MDOH-04 Grown With Cyanobacteria Compared To The Control. Log-transformed colony count data for the cyanobacteria and *Vibrio* growth tubes, as well as the *Vibrio*-only control tubes from day 1 to day 10. All points represent the average of three replicates

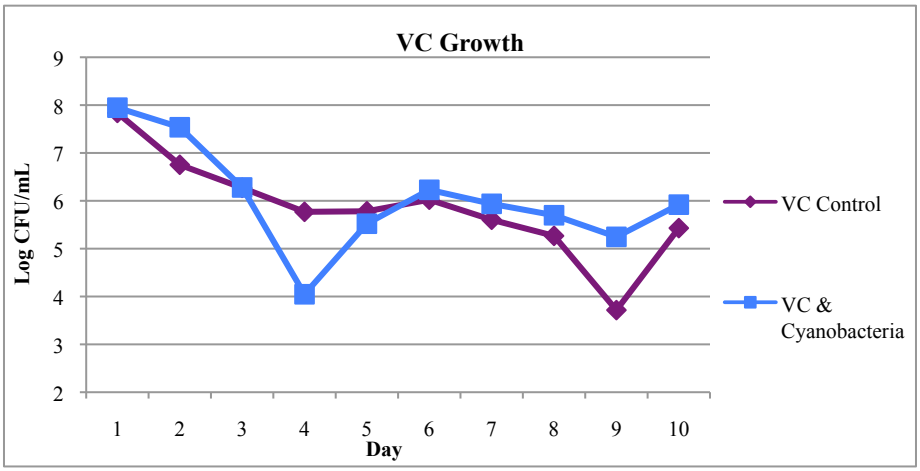

Figure 3 –VC Grown With Cyanobacteria Compared To The Control. Log-transformed colony count data for the cyanobacteria and *Vibrio* growth tubes, as well as the *Vibrio*-only control tubes from day 1 to day 10. All points represent the average of three replicates, with the exception of day 9 and 10 points, which only represent two of the replicates.

3.2 – Is there a difference between *V. parahaemolytics* and *V. cholerae* stains grown with Cyanobacteria?

It was important to compare *V. parahaemolyticus* growth with cyanobacteria to that of *V. cholerae* and cyanobacteria, which is the positive control. It can be seen that G445 grown with cyanobacteria ends at a higher concentration compared to that of the control, despite beginning at similar concentrations (Figure 4). It can also be noted that MDOH-04 behaved similarly to *V. cholerae*, and ended at a cell concentration nearly identical to that of *V. cholerae* (Figure 5). It appears that both MDOH-04 and *V. cholerae* experienced an overall downward trend in abundance whereas G445 experienced the opposite and actually grew to a larger concentration.

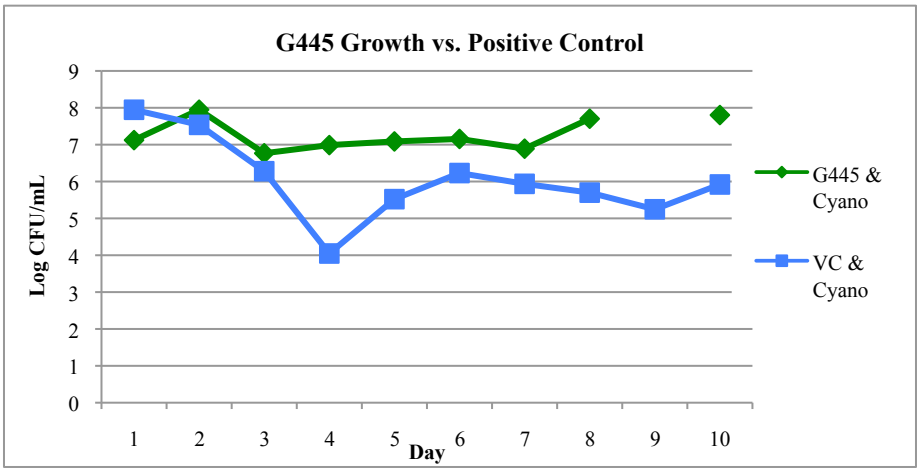

Figure 4: G445 Grown in the Presence of Cyanobacteria Compared to VC Grown With Cyanobacteria. Log-transformed colony count data for the cyanobacteria and G445 growth tubes, compared to the VC and cyanobacteria tubes from day 1 to day 10. All points represent the average of three replicates, with the exception of day 9 and 10 points, which only represent two of the replicates. There were also no countable colonies for G445 and Cyanobacteria for Day 9; the lowest dilution had too few colonies while the next highest had TNTC.

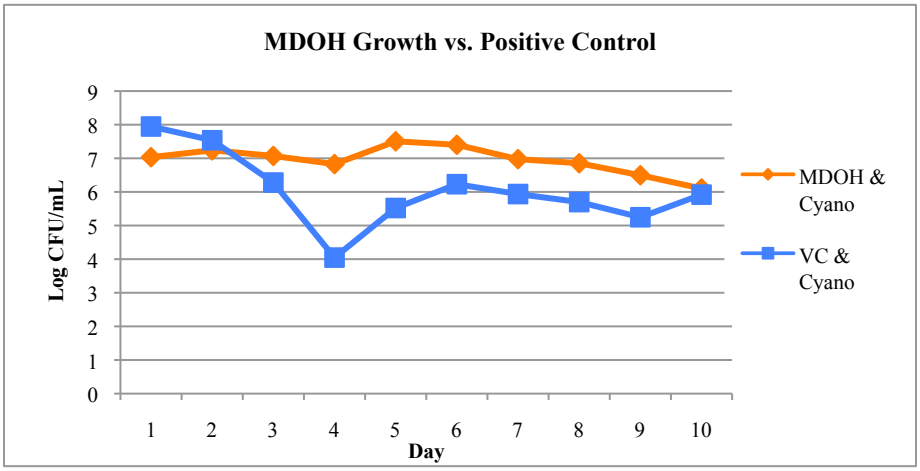

Figure 5: MDOH-04 Grown in the Presence of Cyanobacteria Compared to VC Grown With Cyanobacteria. Log-transformed colony count data for the cyanobacteria and MDOH-04 growth tubes, compared to the VC and cyanobacteria tubes from day 1 to day 10. All points represent the average of three replicates, with the exception of day 9 and 10 points, which only represent two of the replicates for VC.

3.3 – Is there a difference between each of the *V. parahaemolytics* strains grown with cyanobacteria?

There appears to be a noticeable difference between each of the different *V. parahaemolyticus* strains when grown in the presence of cyanobacteria (Figure 6). It can be seen from the graph that the two strains end at very different concentrations. MDOH-04 experienced a decline in overall cell counts whereas G445 appeared to grow and increase in concentration.

Both stains experience similar growth patterns until about day 7, after which they begin to differ greatly.

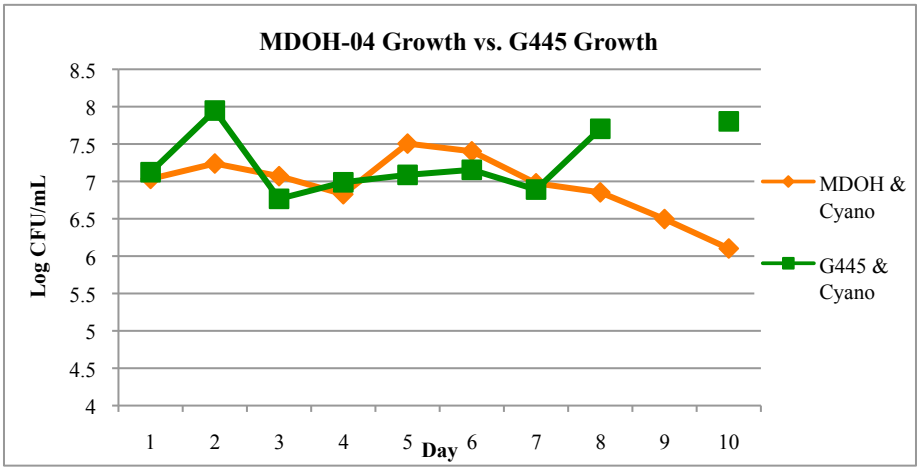

Figure 6: Comparisons Between MDOH-04 and G445 Grown In The Presence of Cyanobacteria. Log-transformed colony count data for the cyanobacteria and MDOH-04 growth tubes, compared to the G445 and cyanobacteria tubes from day 1 to day 10. All points represent the average of three replicates, with the exception of day 9 and 10 points, which only represent two of the replicates for G445. There were also no countable colonies for G445 and Cyanobacteria for Day 9; the lowest dilution had too few colonies while the next highest had TNTC.

3.4 – Are there more culturable cells within the cyanobacterial mats?

It can be noted from the results of the plating of the cyanobacterial mats that *V. parahaemolyticus* strains each experienced a ten-fold increase in the number of cells compared to the concentration at inoculation (Table 1). The original tubes were inoculated with a concentration of 10^6 cells on day 0, so it is important to note this increase in concentration after the mats were broken apart and plated. It is also clear that *V. cholerae* experienced a decrease in the number of cells compared to its original starting concentration, which was also 10^6 cells.

Table 1: Cyanobacterial Mat Plating Results. Calculated CFU/mL values for each *Vibrio* strain after cyanobacterial mats were broken apart and plated.

Strain	CFU/mL Count
MDOH-04 & Cyanobacteria	5.1×10^7
G445 & Cyanobacteria	5.8×10^7
VC & Cyanobacteria	6.1×10^3

3.5 – What do cyanobacteria and *Vibrio* look like under the confocal microscope?

When examining *V. cholerae* grown with cyanobacteria on day 0, the majority of the cells were found in the liquid surrounding the mats; *no V. cholerae* cells were observed within the mats (Figure 7). On day 8, there were still some cells observed in the liquid, but most of the cells were found within the cyanobacterial mats (Figure 8).

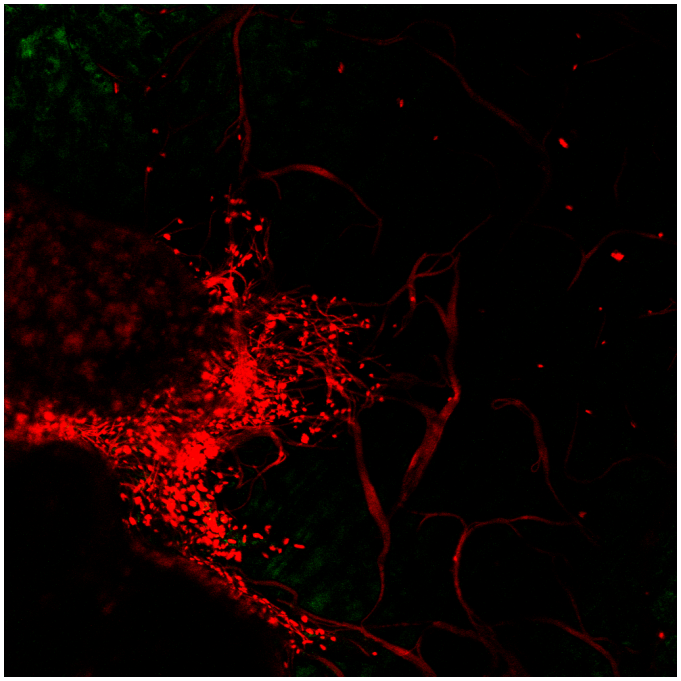

Figure 7: Cyanobacteria and *V. cholerae* Interactions, Day 0.This image captures both Cyanobacteria and *V. cholerae* in an original Day 0 sample.

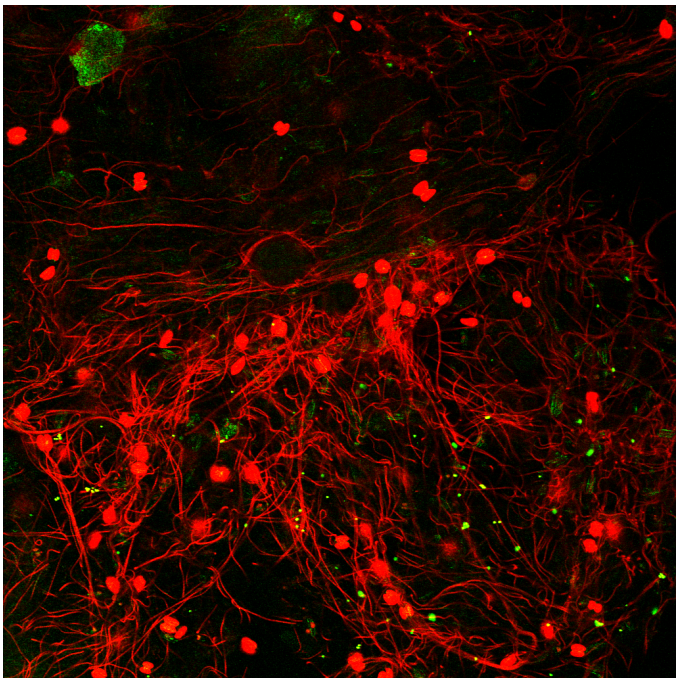

Figure 8: Cyanobacteria and *V. cholerae* Interactions, Day 8.This image captures both Cyanobacteria and *V. cholerae* in an original Day 8 sample.

As seen with *V. cholerae*, there were many G445 cells located in the supernatant of the sample of day 0, and while there were none within the mats, there were a few cells that appeared to be clustering around individual cyanobacterial strands (Figure 9). On Day 8, there was obvious of clustering of G445 cells within the cyanobacteria, seeming to match the grain of the

filaments in the mat (Figure 10). A z-stack (not pictured) showed G445 cells embedded deep within the mats, not simply gathered on the outside.

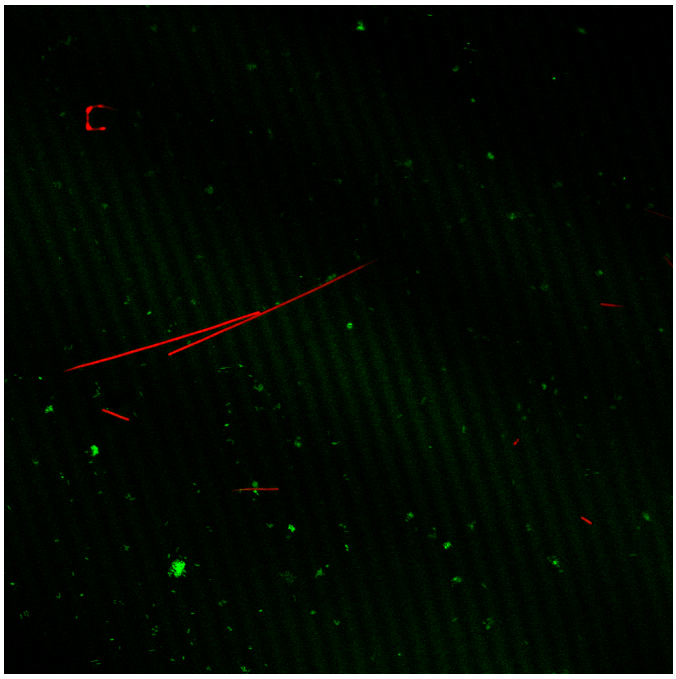

Figure 9: Cyanobacteria and *V. parahaemolytics* G445 Interactions, Day 0.This image captures both Cyanobacteria and *V. parahaemolytics* in an original Day 0 sample.

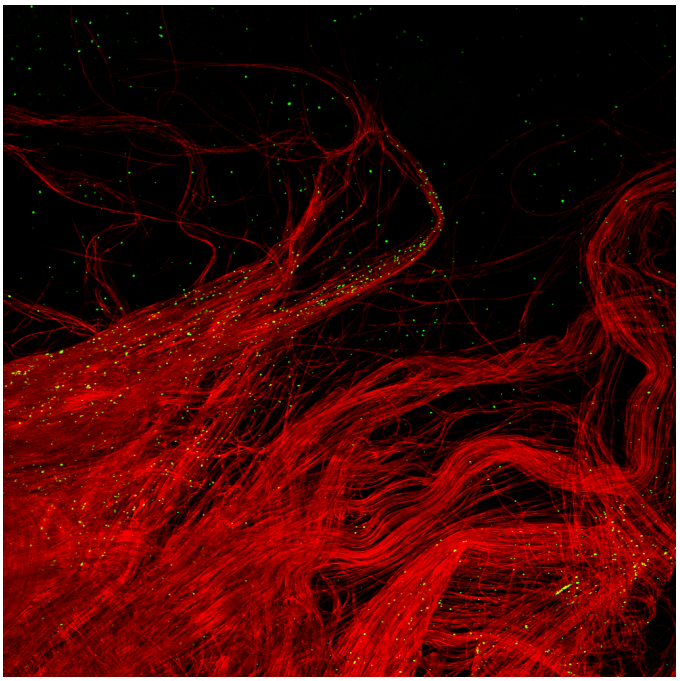

Figure 10: Cyanobacteria and *V. parahaemolytics* G445 Interactions, Day 8. This image captures both Cyanobacteria and *V. parahaemolytics* in an original Day 8 sample.

The final images included are those taken of the negative control beads on day 0 and day 8. It was necessary to include the beads for comparison purposes to the *V. parahaemolyticus* images. Any clustering that is observed in the *V. parahaemolyticus* can be compared to the images of the beads to determine if it is randomized or if the clustering appears purposeful.

All of the observed beads were located in the supernatant and not in cyanobacterial mats on day 0 (Figure 11). When observing day 8, it was seen that there are both beads within the supernatant and the cyanobacterial mats (Figure 12). The beads in the mats however, appear to

have a very randomized pattern and are commonly seen as individual beads on the only on outside of the mats, rather than displaying any major form of clustering as seen in G445.

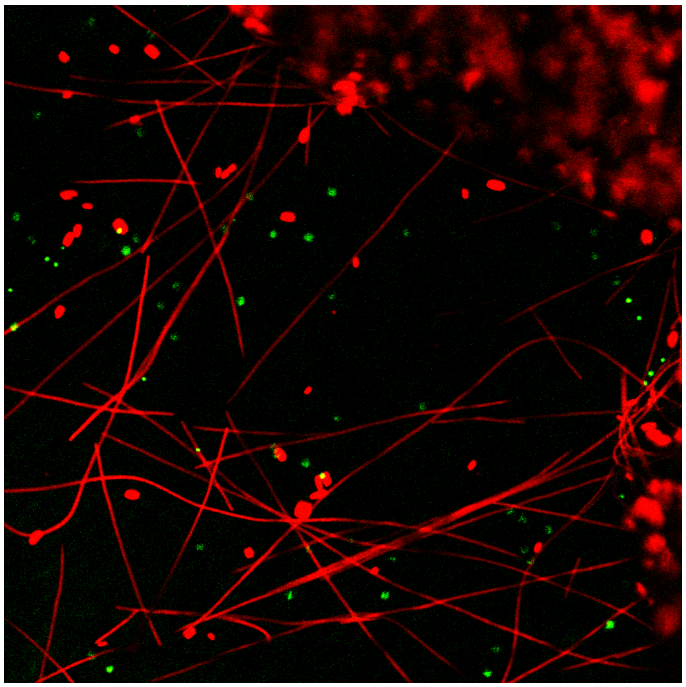

Figure 11: Cyanobacteria and Fluorescent Bead Interaction, Day 0.This image captures both Cyanobacteria and the fluorescent beads in an original Day 0 sample.

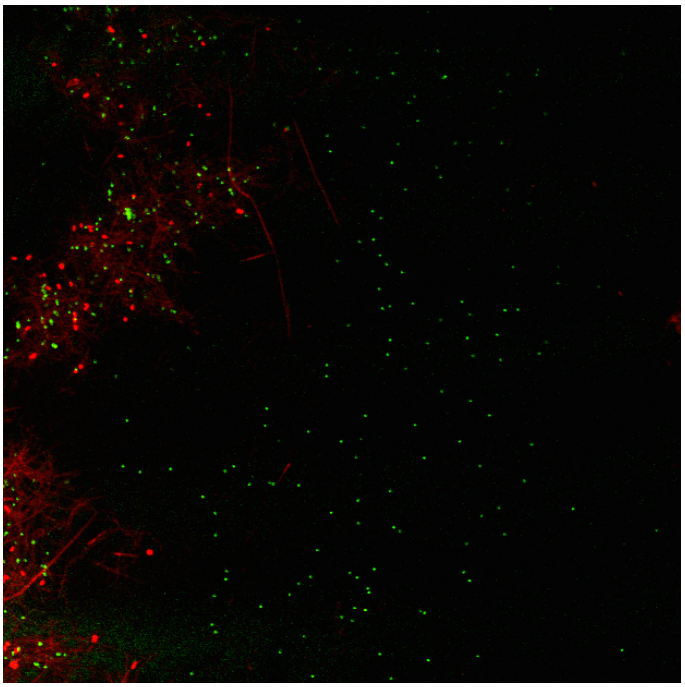

Figure 12: Cyanobacteria and Fluorescent Bead Interaction, Day 0.This image captures both Cyanobacteria and the fluorescent beads in an original Day 0 sample.

4 - Discussion

4.1 - How does each *Vibrio* strain grown with cyanobacteria compare to each *Vibrio* control strain?

The results from the experiment show that MDOH-04 does not persist as long when grown in the presence of cyanobacteria compared to cells grown alone in GBE water. This difference is easily seen when the results are graphed together (Figure 2). The fact that there was a significant difference within MDOH-04 could be contributed to the fact that cyanobacteria were present. There could be an interaction between the two, which affects how MDOH-04 grows. Neither G445 or *V. cholerae* appeared to show a significant difference between the cyanobacteria tubes and the control. Data, however, were missing for each of these strains and a

t-test was unable to be performed to confirm this. Based on a study conducted by Islam *et al.* (2002) we expected that *V. cholerae* to enter a viable but non-culturable state in association with cyanobacteria, which our experiment did not demonstrate. This is most likely because the experiment could not be continued past 10 days. Had plating continued, similar trends to those expected may have occurred.

4.2 – Is there a difference between *V. parahaemolyticus* strains grown with cyanobacteria and *V. cholerae* with cyanobacteria?

Based on the data collected it can be seen that MDOH-04 grown in association with cyanobacteria behaved similarly to *V. cholerae* grown in the presence of cyanobacteria (Figure 6). Both strains ended at a similar final CFU/mL value after the experiment was completed. It can also be observed that G445 when grown in association with cyanobacteria persisted more effectively than *V. cholerae* under the same conditions. In order to confirm if these results are statistically significant a t-test would need to be performed. However, it was impossible to do this as there were some plates that showed colonies out of the countable range and therefore some data was missing for G445. These results provide some interesting insight into the interactions between cyanobacteria and *Vibrio*. *V. cholerae* and MDOH-04, which are both pathogenic, behaved similarly, whereas the environmental G445 behaved differently.

There are many different future experiments that could be performed to test the differences between pathogenic and non-pathogenic strains of *Vibrio* in association with cyanobacteria. It would be beneficial to produce multiple replicates for more effective data collection along with involving a wide variety of strains to see if results hold true on a species level.

4.3 – Is there a difference between each of the *V. parahaemolytics* strains grown with cyanobacteria?

Since there was a difference between how each *V. parahaemolyticus* strain behaved compared to the control, it was essential to compare the two different *V. parahaemolyticus* strains together. These results show a clear difference between pathogenic MDOH-04 growth and non-pathogenic G445 grown in the presence of cyanobacteria (Figure 6). Although a t-test was unable to be performed due to missing data points, it appears from the graph that there is a difference between the two strains. Although there seems to be differences between pathogenic and non-pathogenic *V. parahaemolyticus* grown with cyanobacteria, many more experiments would need to be completed to fully test these results. If similar trends appear in the future, it can be stated with more certainty that there are in fact differences between pathogenic and non-pathogenic *V. parahaemolyticus* strains grown in the presence of cyanobacteria. It would be interesting to figure out what sort of different factors could contribute to differences between the two strains. These could include setting up similar growth tubes but varying the conditions such as light exposure, salinity, and temperature, to see if the results have any major changes. It would also be interesting to perform the experiment with cyanobacteria collected from the vicinity of where this particular MDOH-04 strain was isolated (prior to being a clinical infection). A wide variety of *V. parahaemolyticus* pathogens could also be used to see if there is a pattern, and if so, genotyping or full sequencing could be used to identify common factors among them.

4.4 – Are there more culturable cells within the cyanobacterial mats than the supernatant?

On day 11 of the experiment, the cyanobacterial mats were broken up in GBE water and plated. We speculated that there may be a visible change in colony counts compared to the plates from earlier in the week, due to prior studies observing *V. cholerae* in mats for up to 15 months. It was observed that overall cell counts within the mats went up 10-fold for *V. parahaemolyticus*

strains compared to their starting value of 10^6 cells (Table 1). Both the cyanobacterial mats and the supernatant contained with 10^7 cells on the final day for G445, whereas MDOH-04 only showed an increase in cells from the mats. *V. cholerae* experienced a dramatic decrease in overall cell count over the course of the experiment, decreasing from inoculum at 10^6 to 10^3 culturable cells within the mats. (Table 1). The fact that *V. parahaemolyticus* values actually increased may provide evidence to an idea that cyanobacteria act as a nutrient source for *V. parahaemolyticus*. Previous studies have indicated that cyanobacterial mats may contain a large variety of nutrients, such as glucose, alanine, serine, and aspartic acid, among others (Islam *et al.* 2002). From this information it can be speculated that there may be a possible nutrient exchange between *V. parahaemolyticus* and cyanobacteria. The mats may be able to provide nutrients for the *V. parahaemolyticus* in an otherwise nutrient poor environment. It could also be inferred that *V. parahaemolyticus* are essentially eating the cyanobacteria for fuel. It was noted that the size of the cyanobacterial mats that were in each of the growth tubes dramatically decreased in size over the course of the 9 days of experiments. This observation is also evidence that *V. parahaemolyticus* may in fact be utilizing the cyanobacteria as a nutrient source, which allowed them to prosper.

There are a number of different future studies that could be done to investigate this speculation further. Some prior studies noted that bacteria, specifically *V. cholerae* showed a chemotatic response toward sugars and amino acids within the mucilaginous sheaths of cyanobacteria (Islam *et al.* 2002). A similar experiment could be repeated with *V. parahaemolyticus* to see if results hold true.

4.5 – Are there noticeable interactions between cyanobacteria and *Vibrio*?

There are numerous conclusions that can be reached after observing the images take on the confocal. It can be seen that *V. cholerae* appeared mainly in the supernatant on day 0, whereas on day 8, it was mostly clustered within the cyanobacterial mats. Another important aspect to note is the relatively few cells present in the image compared to the amount of cells that the sample was prepared with. Originally, the day 8 sample was inoculated with 10^6 cells, but cells were rather sparse and did not appear to match the numbers of cells seen in day 0 slides inoculated at the same concentration (Figure 8). This would make sense, as an overall decrease in cell counts via plating was observed over the course of the experiment.

Based on the plate count data and the images taken of the *V. cholerae* sample, it appears that there is something happening within the growth tubes between cyanobacteria and *V. cholerae* that is causing them to decrease in numbers. It is not known at this time what that reason may be for their dramatic decrease, perhaps they are entering a VBNC state as seen in Islam *et al.* (2002). Future studies can surely investigate this topic further.

When observing the images of G445 and cyanobacteria there were various conclusions that were reached. Based on the images taken on day 8, there appeared to be non-random clustering of G445 cells within the cyanobacterial mats. The z-stack (not shown) and other images showed cells deep within the mats on all different levels. There was also a great deal of cells that were grouped together, and comparably few that were non-grouped (Figure 10). These results, along with the increased cell count throughout the course of the experiment, provide evidence that cyanobacteria could be increasing G445 persistence.

This conclusion is also supported by the fact that there were very few fluorescent beads clustered in the mats in the same way. On day 8, it was observed that the beads were not grouped

together the way they were for G445 and there did not appear to be any major stacking within the mat; rather they seemed to be stuck on the outside of the mats. It seems that the few random beads that did end up in the cyanobacterial mats were there because of shaking (Figure 13).

5 –Evaluating the Potential of this Model

Investigating the interactions between cyanobacteria and *V. parahaemolyticus* is an essential aspect to understanding how *V. parahaemolyticus* persists in aquatic environments, such as the Great Bay Estuary. In this study, cyanobacteria were collected from the GBE and grown in association with *V. parahaemolyticus* and *V. cholerae.* The growth of the *Vibrio* overtime was recorded and analyzed for any major changes. The samples were then observed under a confocal microscope to visualize any associations between the two, while using fluorescent beads as a negative control.

Overall this experiment yielded many unexpected and fascinating results, many of which can provide the baseline for future experiments. Based on both qualitative and quantitative data it appears that *V. parahaemolyticus* G445 persisted in the presence of cyanobacteria more effectively than *V. parahaemolyticus* MDOH-04. Another conclusion that can be drawn from this study is that there was a noticeable difference between MDOH-04 grown with cyanobacteria and G445 with cyanobacteria. It was also concluded that MDOH-04, which is pathogenic, behaved similarity to that of pathogenic *V. cholerae*, the positive control. The differences between pathogenic and non-pathogenic strains with cyanobacteria are another topic that has many possibilities for future study, many of which were discussed. Finally, the results suggest that the clustering of G445 *V. parahaemolyticus* within cyanobacterial mats, as shown with confocal microscopy, is not random, and it appears that cells are purposefully entering into these mats.

Differences between G445 and the negative control fluorescent beads provide the reason for this conclusion.

There is obvious value in pursuing this model, and there are also numerous ways that the experiment could be improved upon. One of the first improvements that would be done would be to add back the volume of media that is removed for plating each day to maintain volume levels as long as necessary. Islam *et al*. (2002) conducted growth experiments for an entire month; this could be easily adapted to this experiment by adding back media to maintain volume.

Another change that would be made would be to ensure proper mixing when performing dilutions. It is believed that improper technique could have accounted for some of the variation among the replicates, as well as the major fluctuation over sequential days. It would also be helpful to grow the cyanobacteria in larger quantities. For future experiments it would be beneficial to have more cyanobacteria readily available, enough to include 1g in each sample as performed by Islam *et al.* (2002), or to allow more tubes to be prepared.

Overall if this experiment could be continued in the long term there are many different ways that this model could be expanded upon. It would be very interesting to determine if cyanobacteria could be collected from inside the oyster. Once cyanobacteria samples are taken from inside the oyster, it would be beneficial to see interactions with *Vibrio* under those conditions. Another future investigation would be to determine if cyanobacteria have any genotypic effects on *Vibrio*. This would provide a great deal of insight and evidence to interactions between the two different species. Future research into the interactions between *Vibrio* and Cyanobacteria can hopefully lead to a decrease in the overall number of illnesses caused by the ingestion of *Vibrio* in seafood.

7 – Acknowledgements

I would like to thank several people and organizations for making this undergraduate research experience possible. First, Ashley Marcinkiewicz, who has guided me throughout this project and has always been available to help me in any way possible. Secondly, I would like to thank Dr. Vaughn Cooper, Dr. Cheryl Whistler and Dr. Steve Jones, who welcomed me onto this project and were essential mentors on how to successfully conduct research in a professional setting. I would also like to thank all lab members of both the Cooper and Whistler laboratories, notably Kenneth Flynn, Marcus Dillon, and Jeffrey Sun, all of whom played a role in helping me complete this project. Finally, I would like to thank the University of New Hampshire Hamel Center for Undergraduate Research and Seagrant for funding portions of this project; also the University of New Hampshire Biomedical Science Program, which prepared me immensely for both this research project and my life after graduation.

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9 - Supplemental

ASN III – 1 Liter

NaCl	25.0 g
$MgSO_4 \cdot 7H_2O$	3.5 g
$MgCl_2 \cdot 6H_2O$	2.0 g
$NaNO_3$	0.75 g
$K_2HPO_4 \cdot 3H_2O$	0.75 g
$CaCl_2 \cdot 2H_2O$	0.5 g
KCl	0.5 g
$NaCO_3$	0.02 g
Citric acid	3.0 mg
Ferric ammonium citrate	3.0 mg
Mg EDTA	0.5 mg
Vitamin B_{12}	10.0 µg
A-5 trace minerals	1.0 ml
Distilled water to	1.0 L

pH 7.3±0.2 at room temperature

Trace metal mix A5:

H_3BO_3	2.86 g
$MnCl_2 \cdot 4H_2O$	1.81 g
$ZnSO_4 \cdot 7H_2O$	0.222 g
$NaMoO_4 \cdot 2H_2O$	0.39 g
$CuSO_4 \cdot 5H_2O$	0.079 g
$Co(NO_3)_2 \cdot 6H_2O$	49.4 mg
Distilled water	1.0 L

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