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Quorum sensing: evaluating the effectiveness of diffusion chambers

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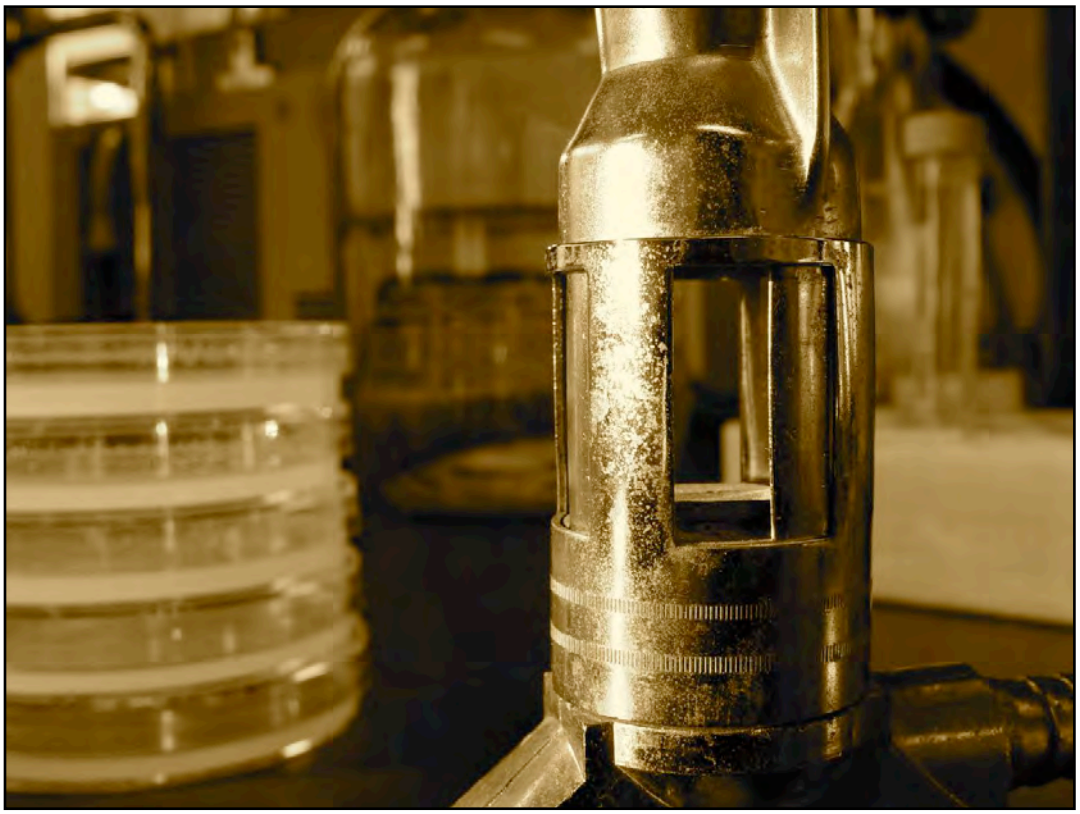
M I C R O B I O L O G Y

Honors Research Project

QUORUM SENSING

EVALUATING DIFFUSION CHAMBERS AS A MEANS OF CULTIVATING NOVEL BACTERIA



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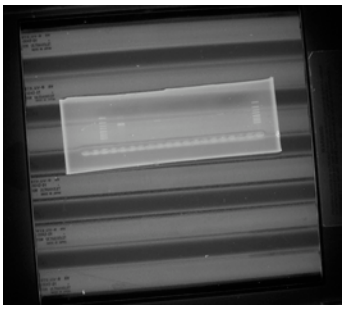
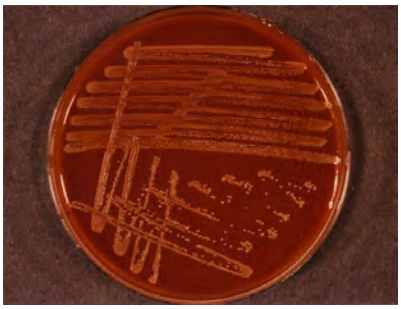
Abstract

Diffusion chambers were evaluated as a means of cultivating previously uncultivable bacteria using enzymatic DNA isolation, PCR amplification with phyla-specific primers, and gel-electrophoresis. The bacterial diversity elucidated by the molecular techniques performed on the diffusion chambers was then compared to samples from the same environment that were grown in agar pour plates. The ultimate goal of this research would be to learn more about bacterial evolution and possibly use newly cultivable microbes to develop new antibiotics, to which bacterial resistances have not yet been developed. In this experiment, a multitude of obstacles presented themselves that prevented reliable results from being garnered and analyzed. As a result, the *Materials and Methods* and *Discussion* sections of the following paper are in-depth looks at the problems encountered during this experiment and the attempts of my lab supervisor, Annette Bollmann, and I to overcome them.

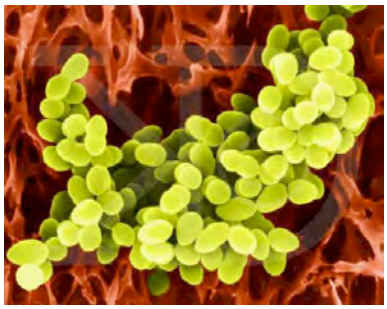
Introduction



Picture of Gel Electrophoresis Product



Bacterial Growth on Blood Agar Plate



Staphylococcus Picture from Microscopy

Project Focus and Overview

The field of microbiology has evolved greatly over the last one hundred years. Since the invention of the microscope, scientists have continued to gain and utilize knowledge about the microbial world that exists ubiquitously around us. However, as scientists learn more about microbial life, it is becoming apparent that we may only be scratching the surface when it comes to the amount of microbial diversity in nature. Since the introduction of molecular techniques to microbiology, new species, families, and even phyla within the bacterial kingdom have been discovered. Up to now, cultured representatives of only half of these phyla are available in the lab (Rappe & Giovanonni, 2002).

There have been many hindrances to the identification and investigation of new microbial life. Arguably, the greatest obstacle is what is commonly known as "The Great Plate Count Anomaly" (Madigan et al., 2002) This theory asserts that nearly 99% of the microorganisms cannot be cultivated in the laboratory because microbiologists cannot create the conditions found in nature that the bacteria require to thrive. Fortunately, it appears that diffusion chambers may help solve this problem (Kaeberlein et al., 2002). Diffusion chambers are essentially two washers that are covered with a permeable membrane. The diffusion chambers are then placed in the environment and contain media concordant to their surroundings. Their permeability allows nutrients and growth factors to pass between the environment and the chamber. Thus far, these chambers have been

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able to help cultivate the novel bacteria with a great deal of success. However, a direct study of this phenomenon has not yet been performed. The goal of my project is to evaluate whether or not the diffusion chambers display a statistically significant increase in microbial growth compared to agar pour plates that were created in lab from common soil samples. It is my contention that there will be a greater diversity of phyla in the diffusion chambers than the agar pour plates.

Materials and Methods

Note. My experiment failed to yield significant results and was inconclusive, at best. In order to try and garner some results my lab supervisor Annette and I tried modifying different aspects of my protocols several times in order to create a procedure that would repeatably produce data that could be further analyzed. The following section of my report is a detailed recount of our attempts to overcome these issues and the materials we used to try and do so. All of the following protocols are listed in the *Protocols and Tables* section of the report for reference and clarification of any confusion.

Sample collection. Fresh water sediment samples were collected from the turtle pond of Hyde Park in South Boston, MA. These samples were collected on 10.1.04 and identified as the first generation *A1 10.1* sample set. The samples were allowed to incubate from 10.1.04 until 11.8.04 and were then divided into two second generation diffusion chambers entitled *A1-1 11.8* and *A1-2 11.8*. Sediment from these second generation diffusion chambers was then transferred to agar pour plates and third generation diffusion chambers on 12.4.04. This procedure was repeated to create the fourth generation diffusion chambers and generate a new set of post-third generation agar pour plates on 1.27.05. Lastly another set of agar pour plates were created from samples taken from the fourth generation diffusion chambers on 3.3.05. This entire procedure was repeated from the following environmental samples: *A2 10.1*, *CY1 10.1*, *CY2 10.1*, *CYE1 10.1*, and *CYE2 10.1*.

DNA Extraction. DNA isolation was performed enzymatically using the Qiagen DNeasy isolation kit. As a result of my inability to garner results, I used two slightly different protocols in hopes of extracting DNA. In both protocols, the sample material was homogenized using a 3cc syringe and 25 gauge needle. Material was transferred from the provided tubes to the syringe using plastic sterile loops. The homogenized material

was placed in 2mL Eppendorf tubes and marked as their respective sample name with a subscript "s" to denote that it was the homogenized stock.

DNA Isolation 1. The first DNA isolation protocol was derived from the Qiagen DNeasy Handbook for the isolation of genomic DNA from Gram-negative bacteria. For each individual sample, 90µL of stock was pipetted into a 1.5mL Eppendorf tube to which 360µL of Buffer ATL and 40µL of Proteinase K was added. The reactants were then vortexed for fifteen seconds and incubated at 55˚C for three hours. After incubation, the reactants were vortexed before and after the addition of 400µL of Buffer AL to the tube. Once again the samples were incubated but this time for ten minutes at 70˚C. Afterward, 400µL of 96-100% Ethanol was added to the sample and vortexed.

Once the DNA was exposed, it needed to be isolated from cellular components and debris. This was accomplished by pipetting 700µL of the exposed DNA into a 2mL DNeasy spin column provided by Qiagen. The mixture was centrifuged for one minute at 8,000 rpm through the spin column, which bound the DNA from the mixture as it passed through. This was repeated until all of the sample passed through the spin column. Next 500µL of Buffer AW1 was added and centrifuged for another one minute at 8,000 rpm to wash the column of any debris still attached to the DNA. Next, 500µL of Buffer AW2 was added to the column and centrifuged for three minutes at 13,200 rpm to ensure the removal of ethanol from the sample. The DNeasy spin column was then transferred to a new 1.5mL Eppendorf tube and 200µL of Buffer AE was added and allowed to sit for one minute. The Buffer AE was then centrifuged for one minute at 8,000 rpm in the DNA elution step. This was repeated one more time to generate a total of 400µL of sample containing the DNA. The tubes were then labeled with their respective name and the subscript "i" to denote they were the isolate and stored in the box labeled 'Nathan Chamber/Pour Plate Material' and placed in the 4˚C freezer.

DNA Isolation 2. The first protocol was not generating results, so Annette suggested I try using a different protocol that utilized the Qiagen kit and had worked in the past. First, a DNA isolation buffer had to be created. The buffer was constituted of 20mM Tris HCl, 2mM EDTA, 1.2% Triton and 20mg/mL of lysozyme. Once the buffer was created, 440µL of it was combined with 110µL of stock and vortexed for fifteen seconds. After, the samples were incubated at 37˚C for three hours. Then 75µL of Proteinase K and 610µL of Buffer AL were added and vortexed before being incubated for an additional thirty minutes at 70˚C. After the incubation step, 610µL of 96-100% ethanol was added. The spin column process was repeated with the sample solution as it was in the first isolation, except the elution step used only 50µL of Buffer AE in each individual

elution, in total equaling 100µL of isolate per sample. This was done in hopes of acquiring a greater concentration of DNA in the elution.

DNA Clean-Up. This step was also added in order to try and generate results. We believed that there may have been some inhibitors present in the stock that were preventing isolation or possibly the PCR reaction. The protocol followed is derived from the Promega Wizard® DNA Clean-Up System. First, the provided DNA Clean-up Resin was thoroughly mixed and warmed to 37˚C to dissolve any crystals present in the resin. The resin was then mixed and 1mL of it was placed into a 1.5mL microcentrifuge tube. After, 100µL of the isolate generated from the *DNA Isolation 2* protocol was added to the tube and mixed by inverting the tube several times. The contents of the tube were then pipetted into a 3cc syringe with a Leur-Lok® minicolumn extension attached to the end to bind the DNA. Slowly, the plunger of the syringe was pressed and the flow-through was discarded into the waste bucket. The minicolumn was then detached and 2mL of 80% isopropanol was pipetted into the syringe. The minicolumn was then reattached to the syringe and the isopropanol was passed through it. The minicolumn was once again removed from the syringe and transferred to a 1.5mL microcentrifuge tube. The tube was then centrifuged for 2 minutes at 10000 rpm to dry the resin. The minicolumn was transferred to a new microcentrifuge tube and 50µL of TE buffer (diluted to 10mM Tris-HCl, 1mM EDTA) was placed in the minicolumn and allowed to sit for one minute to provide adequate absorption. The minicolumn was then centrifuged for twenty seconds at 13,200 rpm to elute the DNA. The waste was thrown away and the purified DNA was stored in the 4˚C in the box labeled 'Nathan Chamber/Pour Plate Material'.

Agar Inhibition. After the first two DNA isolation procedures did not lead to the generation of results, we used β-agarase to try and destroy any inhibitors that the Wizard® DNA Clean-Up System may have been missing. This step had to be performed prior to the isolation, so we started over with fresh homogenized samples. We chose to analyze samples A1, A1-1 10.1 and A1-2 10.1. After combining 100µL of the sample agar and 11µL of the provided β-agarase reaction buffer, an additional 2µL of the β-agarase was added and incubated at 42˚C for one hour. Then, the β-agarase was deactivated by placing the tube in the 65˚C hot plate for fifteen minutes. The *DNA Isolation 2* protocol was then followed in conjugation with the clean-up steps.

PCR Amplification. The PCR was was performed by first creating a PCR mix:

PCR INGREDIENTS	DESIRED RATIOS (MICROLITERS)
H_2O	18.75
Buffer	2.50
Primer (reverse)	.50
Primer (forward)	.50
dNTPs	.50
BSA	1.00
Taq Polymerase	.25
DNA Sample	1.00
Total	25.00

This mixture is generated with these ratios multiplied by the number of samples (including positive and negative controls) needing to be processed plus one additional milliliter to account for any errors or extra solution that may be needed. All of the ingredients were added using filter tips, except for the DNA samples. Once everything was combined, the mixture was vortexed to ensure uniformity in distribution of the reactants. Subsequently, 24µL of the mix was placed into PCR tubes and 1µL of DNA was added to each one. The samples were then placed into the PCR machine and set to the "col30" setting that repeated the amplification step thirty times and had an annealing temperature of 50˚C. As a result of the issues we were having, we changed amplification repeating step to thirty-five cycles at one point and changed the annealing temperature to 52˚C with no success. We also used different primers in hopes that one set would be more successful than the general F27-R1492 primers we were using. We tried F27-R1090, F502-R1492, F502-R1090, F27-R1368, and F502-R1368. None of these combinations was successful in yielding a product that could be visualized on the gel.

Gel Electrophoresis. In order to determine whether DNA was present in our samples, we had to run our PCR products on a gel. First, a 1% agarose gel had to be created. One gram of agarose was added to 100mL of TE Buffer. The agarose was mixed into the buffer and microwaved for three minutes. Once the agarose was dissolved in the buffer, it was poured into the gel mold with lane combs and allowed to sit for thirty minutes to solidify. Once solidified, the lane combs were removed, leaving lanes to place our samples in. Samples were prepared for the gel by placing 5µL of loading dye onto parafilm and adding 1.5µL of the DNA sample to it. Then, the samples were pipetted into the lanes of the gel. In the last lane, a 1kilobase ladder was a added in order to

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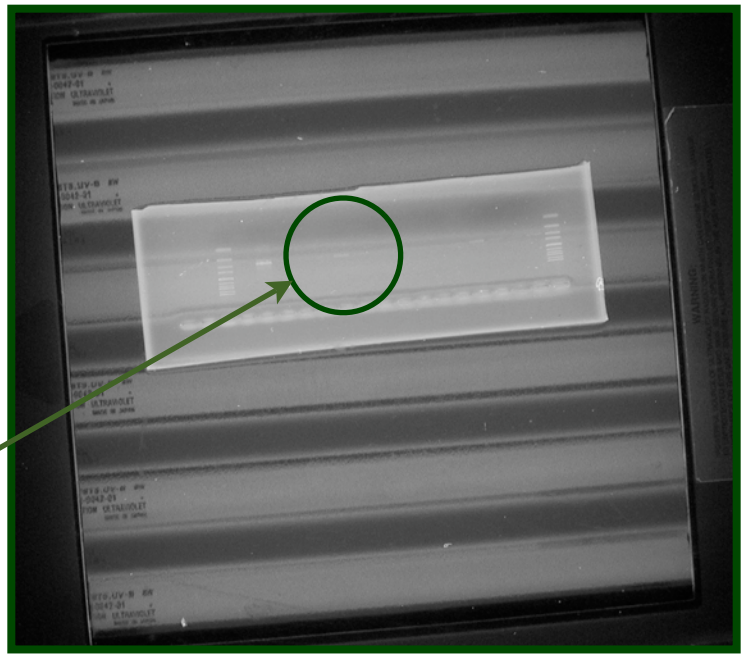
give reference to the migratory distance of the bands. The gel was run at a constant voltage of 120V and for a time of 30 minutes.

Results

The results that were generated in this experiment were generally non-existent or inconclusive. There was a multitude of obstacles that were encountered that can be reviewed in the *Discussion* section and our attempts to adjust the protocols to overome these obstacles can be seen in the *Materials and Methods* section.



For the most part, gels appeared like the one to the right. The ladders were clear and the positive control was bright but other bands were non-existent or really faint.

Protocols and Tables

DNA Isolation 1
Isolation of Genomic DNA from Gram-negative bacteria

I. Homogenization of Material

- Remove syringe plunger
- Attach capped 25G needle to syringe

- Use plastic sterile loop to transfer material
- Push through needle into 1.5mL Eppendorf tube
- Stock is created.

II. Expose DNA

- Put 90µL of stock into 1.5mL Eppendorf tube
- Add 360µL Buffer ATL
- Add 40µL Proteinase K
- Mix by vortexing for 15s
- Incubate @ 55˚C for 3 hours or overnight; vortex occasionally
- Vortex for 15s
- Add 400µL of Buffer AL, vortex thoroughly (15s)
- Incubate @ 70˚C for 10 minutes
- Add 400µL of pure Ethanol and vortex

III. Isolate DNA

- Pipet 700µL mixture into 2mL DNeasy Mini spin column
- Centrifuge for 1 minute @ 8,000 rpm; discard waste
- Repeat until all sample is passed through spin column (keep tube)
- Place the Dneasy spin column in new 2mL tube
- Add 500µL of Buffer AW1
- Centrifuge for 1 min @ 8,000 rpm; discard waste
- Place the Dneasy spin column in new 2mL tube
- Add 500µL of Buffer AW2
- Centrifuge for 3 minutes @ 14,000 rpm (removal of ethanol)
- Place the DNeasy spin column in new 2mL tube
- Add 200µL of Buffer AE, let sit one minute
- Centrifuge for 1 minute @ 8,000 rpm
- Repeat elution
- Combine elutions (400µL total)*

*decreases DNA concentration but increases overall DNA yield

IV. Storage

- Place samples into box labeled 'Nathan', place in fridge.
- Place stock samples into freezer boxes 'Chamber' or 'Pour Plate'

DNA Isolation 2
Isolation of Genomic DNA from Gram-negative bacteria

I. Buffer Creation

- 20mM TrisHCl
- 2mM EDTA
- 1.2% Triton
- 20 mg/mL lysozyme

II. Isolate DNA

- Place 440µL of buffer and 110µL of homogenized bacteria sample into 2.0mL Eppendorf tube.
- Incubate samples for three hours at 37˚C
- Add 75µL of Proteinase K
- Add 610µL of Buffer AL
- Vortex and incubate for 30 minutes at 70˚C
- Add 610µL of 96-100% alcohol and vortex
- Put 600µL on spin column and elute at 8000 rpm for 1 minute
- Discard flow-through and repeat two times
- Wash with 500µL of buffer AW1 and AW2 at 8000 rpm for 1 minute and 13200 rpm for 3 minutes, respectively
- Elute with 50µL of buffer AE two times
- Collected elutions are the isolated DNA

IV. Storage

- Place samples into box labeled 'Nathan', place in fridge.
- Place stock samples into freezer boxes 'Chamber' or 'Pour Plate'

PCR Amplification
Amplification of Genomic DNA from Gram-negative Bacteria

Procedure

I. Create PCR Mix:

PCR INGREDIENTS	DESIRED RATIOS (MICROLITERS)
H_2O	18.75
Buffer	2.50
Primer (reverse)	.50

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PCR INGREDIENTS	DESIRED RATIOS (MICROLITERS)
Primer (forward)	.50
dNTPs	.50
BSA	1.00
Taq Polymerase	.25
DNA Sample	1.00
Total	25.00

Important Notes:
Total for our Experiment= (Desired Ratio) x (Number of Samples +1)
Vortex Mixture with DNA
When adding DNA, do not use filter tips

II. Place PCR Mix with DNA into Rows and label

III. PCR Machine Procedure (Commands)

a. One cycle
b. Place samples in machine
c. User-Annette
d. Enter
e. Protocol Lib
f. Col PCR30
g. Run protocol
h. Left or Right->
i. Hot
j. Sample Volume-> 25μL
k. Begin Run

IV. PCR Procedure

STEP 1	STEP 2A	STEP 2B	STEP 2C	STEP 3	STEP 4
95˚C	95˚C	50˚C	72˚C	72˚C	4˚C
5.00min	1.00min	1.00min	1.00min	10.00min	Storage

*steps 2a, 2b, and 2c repeat themselves 30 times.

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Gel Electrophoresis
Gel Electrophoresis of PCR Products from amplified Genomic DNA originating in Gram-negative Bacteria

Gel Electrophoresis Procedure:
I. Gel Creation
- a. Desired 1% agarose in gel. (1mL=1mg of agarose)
- b. Mix powder and water for 3 minutes
- c. Place in desired mold with comb
- d. Let sit 30 minutes
- e. Cut excess with razor in step-wise fashion
- f. Put gel into electrophoresis machine
- g. Ensure origin is close to black electrode

II. Creating Gel samples from PCR Products
- a. Create a 2″x2″ square of parafilm
- b. Place 1.5µL of loading dye on parafilm x # of samples
- c. Place 5µL of PCR product on each spot
- d. Place 5µL of marker on each spot
- e. Pipette into gel

III. Run gel at 120V for 30 minutes

Discussion

As mentioned, the experiment failed to yield any results. This discussion is a look at the problems encountered and other factors that may have contributed to my inability to isolate DNA and display it on a gel.

Technical Issues. There were many technical issues we tried to address in order to create a protocol that would repetitively yield results. The *DNA Isolation 1* protocol was unsuccessful in the first half of the experiment, so we began using the *DNA Isolation 2* protocol that had worked for Annette Bollmann in the past. When the new method of DNA isolation failed, Annette and I postulated there may be inhibitory substances in

the isolate that were preventing the PCR reaction from taking place. We then used the Promega Wizard® DNA Clean-up system in hopes of eliminating the inhibiting factors. When that didn't work, we also used β-agarase in hopes of breaking down the agar and any other possible factors that may have been affecting our results. Because we tried various isolation methods, we began to postulate that perhaps different sets of primers would be more effective in amplifying the DNA in the samples. We used the six different combinations of primers listed in he to no avail. After discussing the problem further with Annette, we decided that perhaps there was too much DNA in the isolate and that it may have inhibited the Taq polymerase from amplifying the sample. We then performed several dilutions, in order to determine the most effective dilution for PCR amplification. Dilutions included 1:10, 1:100, and 1:1000 of the original isolate samples. Of the three the 1:100 generated bands, however they were very faint and I had trouble duplicating them.

Human Error. The other factor that must be addressed is human error. Being an undergraduate, this was my first major exposure to microbiology research and the molecular techniques we used. As I was unable to ascertain why my samples would not yield results, I was forced to look at myself as the cause. However, I did have Annette supervise one complete isolation procedure and she was unable to determine what errors I may have been responsible for. The only other issue that presented itself was a lack of time to work on the project. During the first semester I was able to get the protocols to work for some samples that were provided to me and had planned on beginning the research on my specific samples during the spring semester. However, for two and a half weeks the lab was unavailable to work in because it was being moved to a new renovated room in Mugar Hall. However, I do not believe this was a significant problem and I made up for this lost time by spending a much greater amount of time in the lab later in the semester.

Summation. Overall this research project and my experience in the lab was extremely beneficial to me despite the lack of results. I believe the exposure to a lab environment and the workload associated with it will greatly benefit me in the next stage of my career when I will be conducting research at the National Institute for Neurological Disorders and Stroke for fifteen months. I also gained valuable laboratory experience and added useful molecular techniques to my skill set. In hindsight, I was very fortunate to be able to gain this experience and learn about laboratory research. If I had it to do over again and knew I would not get results in advance I would still do it for the research experience and familiarity I gained with a laboratory environment.

Acknowledgments

Despite the lack of results in this experiment, I learned a great deal about microbiology and became familiar with several molecular techniques in my time in the lab. Even though everything did not go as I had planned, the support and patience of the members of the lab was greatly appreciated, specifically lab supervisor Annette Bollmann and Professor Epstein. I am very grateful for their assistance and willingness to take on an undergraduate in their laboratory.

I would also like to thank those in the honors department for their assistance and the hard work they put into this program so students such as myself can have these kinds of opportunities. I know this project has helped prepare me for the next step of my career by providing me with first-hand experience in research science to complement the information I have learned in the classroom. Once again, thanks to everyone that made this project possible and that have helped me along the way.

Works Cited

Madigan, Martiko, and Parker. Brock Biology of Microorganisms. London: Prentice Hall, 2002.

Henckel, T., M. Friedrich and R. Conrad. 1999. "Molecular Analyses of the Methane-Oxidizing Microbial Community in Rice Field Soil by Targeting the Genes of the 16S rRNA, Particulate Methane Monooxygenase, and Methanol Dehydrogenase." Applied and Environmental Microbiology. 65: 232-242.

Rappe, M. and S. Giovannoni. 2003. "The Uncultured Microbial Majority". Annual Review of Microbiology. 57: 369-394.

T. Kaeberlein, K. Lewis, S. Epstein. 2002. "Isolating 'Uncultivable' Microorganisms in Pure Culture in a Simulated Natural Environment. Science.296: 1127-1129

Stach et al., 2003. Nucleic Acids Residue. Science 31: e2.