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Occurrence of Multiple Antibiotic Resistant Bacteria in Aquatic Environments in Central Minnesota

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Occurrence of Multiple Antibiotic Resistant Bacteria in Aquatic Environments in Central Minnesota

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In Partial Fulfillment of the Requirements for Distinction in the Department of Biology

By

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Abstract:

Antibiotic resistance among bacteria has been a topic of concern for many years. The purpose of this study was to evaluate the occurrence of antibiotic resistance among bacteria found in aquatic environments such as the lakes on the campus of Saint John's University along with the Sauk River in Cold Spring and St. Joseph, Minnesota. By analyzing samples from different aquatic environments, this data can be used to better identify patterns of resistance within different genera of bacteria. A total of 125 isolates were captured from these different areas and isolated into pure cultures. The isolated cultures were grown on agar plates made with a fixed concentration of antibiotic, inoculated with antibiotic disks placed on DB agar plates, and 96 well plates filled with increasing concentrations of antibiotic. Twenty-six isolates were chosen to pursue based on their resistance levels to five or more antibiotics. A series of standard microbial tests were done along with PCR of the 16S ribosomal RNA protein to identify these bacteria and almost all were gram negative. The cultures represented 7 different genera with Flavobacteria and Acinetobacter being the most common. Resistance coefficients were calculated based on optical density values relative to cells grown without antibiotics in the well. This study suggests multi-resistant, gram-negative bacteria are common in aquatic environments in central Minnesota, which presents interesting questions about the (over)-use of antibiotics. This information will likely aide in attempts to limit antibiotic consumption by providing information about patterns of resistance in different genera of bacteria.

Introduction to antibiotic resistance:

Since the beginning of the use of antibiotics in the 1930s, specific mechanisms have been created by bacteria to put an end to their effectiveness. It has been estimated that since the introduction of antibiotics there have been "millions of metric tons of antibiotics" produced for different purposes including medicinal and agricultural (1). This amount of release into the environment has increased since their introduction, which produces new environmental pressure for the bacteria to create new proteins/mechanisms against these antibiotics. One way to see the increase in resistance is that the baseline minimum inhibitory concentration (MIC) of antibiotics is slowly getting higher (2). MIC can be defined as the lowest concentration of antibiotic that visibly inhibits the growth of bacterial colonies. Therefore, as time goes on, it is taking a higher concentration of these antibiotics to kill these bacteria (3). There has been increased use of antibiotics not just in treating infections in hospitals, but also used in agriculture and animal/fish farms. Due to these different uses, different communities of bacteria may have been selectively pressured to be able to survive these higher concentrations and therefore have created resistance mechanisms (4). This becomes a large issue in the future of their use for a dependent society.

Most research on antibiotic resistance has been concentrated on the clinically important pathogenic bacteria because that is what is most important to society (5). However, over the past decades, it has been noticed that environmental bacteria may act as a reservoir of antibiotic resistance mechanisms that may be able to be transferred to pathogenic bacteria (6). Therefore, natural environments such as lakes and streams have become targets for antibiotic resistance testing. Through the years there has been heightened awareness of superbugs becoming more prevalent and more dangerous within communities. This means there is an increased need for different antibiotics that will attack these already resistant organisms, making drug manufacturers' jobs that much more time consuming and difficult. One key thing to look at here is that within the ecosystem, the organisms producing the antibiotics that pharmaceutical industries are interested in may play a large role within their own communities. The small amount of antibiotic given off by these producers may be enough in fact to change transcription among the bacteria in its community to be resistant to that particular antibiotic and therefore increasing resistance levels of the community (3). It has also been found that for these bacteria that live in aquatic environments, having a mechanism to protect against antibiotics proves to be beneficial against other charged waste or as a type of signal among its community (6). These mechanisms of resistance are used almost as a defense mechanism and are the reason these bacteria can form essentially a reservoir of resistance genes (7). It is also important to note that if there are resistance mechanisms on "mobilizable" bacteria, their ability to transfer these mechanisms to different genera is greatly increased, potentially creating multiple communities of resistant bacteria (7). Bacteria can be resistant to more than one antibiotic without being pathogenic (8). As a result, limited families/classes of antibiotics used in fighting common infections may be ineffective. This poses a problem because research has shown that mechanisms a bacterium may have against one antibiotic may have similar activity against

another antibiotic in the same class (9). With limited options in terms of classes of antibiotics, multiple antibiotic resistance is becoming more common.

This increased level of antibiotic resistance and the interaction among bacteria can be attributed to both the natural environment and human overuse of these antibiotics for infections. The mere presence of antibiotic resistance in bacteria means danger to the future of antibiotics due to bacteria's ability to transfer genes (most importantly, antibiotic resistant genes) now known as horizontal gene transfer (10). The fact that bacteria are able to transfer genes quickly and efficiently is something that stands in the way of being able to effectively overcome antibiotic resistance. This antibiotic resistance has been related to high selection pressure for these bacteria with mutated genotypes (having resistance mechanisms) when there is antibiotic present in the environment for an extended period of time (11). There have been multiple attempts to combat this resistance by pairing antibiotics together such Sulfamethoxazole-Trimethoprim (SXT) in order to treat infections. This solution, however, is only a temporary fix for this problem which society now faces. The reason antibiotic resistance is such an important issue lies in the fact that society today is completely dependent upon antibiotics to treat infections and without them, things like surgical operations and other organ transplants would become less successful. Without the proper recognition it deserves, antibiotic resistance will eventually lead to an era similar to the pre-antibiotic era.

In terms of antibiotic resistance, there are multiple levels to look at when considering this problem. One question that poses to be an issue is how resistant are the bacteria currently living in the lakes and streams of our state? If resistance to antibiotics occur, is there a pattern present among antibiotics or among genera of bacteria? The answers to these questions are important because it will give insight into the amount of resistance within aquatic environments under different environmental pressures. Things such as the closeness of farms, factories, and human activity all play roles in the antibiotic resistance within the bacterial community of these aquatic environments. With this knowledge we can better identify patterns of resistance within different genera of bacteria.

We took it upon ourselves to study aquatic bacteria and the level of multi-resistance present in lakes and rivers around the college campus of Saint John's University and St. Cloud. Using different antibiotic classes in DB media (9g Bacto Agar and 0.6g Tryptone per 600mL of water), the resistance of different isolates from different aquatic areas was measured. This data then can be used to enhance the knowledge of the current antibiotic resistant situation among aquatic bacteria in areas not subject to high amounts of antibiotics. It is important to know this information because it will aide in the attempts of controlling antibiotic consumption knowing the resistance levels of bacteria just in lakes and streams. It will also help in giving information about the genera of bacteria that are most resistant and how resistant they actually are to different classes and concentrations of antibiotics. If these bacteria are able to become resistant in low concentrations, imagine the amount of resistance among bacteria closer to environments where antibiotics are emitted.

Methods:

Sampling and Purifying:

There were 17 different locations sampled throughout the lakes of Saint John's University and the Sauk River in Cold Spring and Saint Joseph, Minnesota. Of the lakes on the Saint John's University campus, two samples (labeled B and C) were taken from East Gemini Lake, two samples (labeled G and H) from West Gemini Lake, two samples (labeled A and F) from Stumpf Lake, and two samples (labeled D and E) from Lake Sagatagan. Then three samples (labeled I, J, and K) were taken from the Sauk River in Cold Spring. Sample I and J were taken in downtown Cold Spring with I being near a park and J being on the side of a bridge used for fishing. The sample K was upstream of a chicken farm. Two samples (labeled L and M) were taken down river from agricultural farms. These samples were around a chicken farm to test levels of antibiotic run-off in order to assess overall effect on bacterial community (of possible increase in environmental pressure). The other four samples (labeled N, O, P, and Q) were taken from the Sauk River in St. Joseph, Minnesota, near a frisbee golf course. Each sample of water was plated out onto DB agar plates. For each sample, two plates were made, one with 50 µl of the sample water distributed in the middle of the plate, the other with 100 µl of a mixture of 100 µl of sample and 900 µl of dilution broth to dilute it. These plates were then incubated for 48 hours at room temperature. Once the plates had grown sufficiently, then roughly 10 colonies were selected from each location for isolation using sterile toothpicks for a transfer tool. Each colony selected was touched with the toothpick, then streaked out four times onto a new DB agar plate. These were left alone to grow for 48 hours. Each colony was then purified through a streaking technique using a sterile loop and incinerator, onto new DB agar plates to ensure pure colonies. After purification, colony morphology was recorded.

Testing:

Each of the 125 colonies were tested against 8 antibiotics. These antibiotics include: streptomycin sulfate, penicillin G., ampicillin, tetracycline, neomycin sulfate, erythromycin, nalidixic acid, and amoxicillin. To do this, each antibiotic was added to the DB agar during the pouring process. After the agar had been autoclaved, 0.04g of each antibiotic was measured out and placed into separate agar flasks when they were cooled to 60 degrees Centigrade, giving a final agar concentration of 66.7mg/mL. Once the plates were poured and solid, each of the isolates was tested. On each antibiotic plate, using sterile toothpicks, each colony was touched and streaked onto the plate allowing four colonies to be streaked per plate. Once all colonies had been streaked out on the eight different antibiotic plates, these were then put in a room temperature incubator for 48 hours. The results were then recorded based on amount of growth, color, and whether it had swarming capabilities for each colony.

Each colony was then tested against antibiotics in disks soaked with differing concentrations of a particular drug. These antibiotics include: cephalothin (30 µg), sulfamethoxazole trimethoprim,

bacitracin (10 μg), nitrofurantoin (300 μg), imipenem (10 μg), and amoxicillin/clavulanic acid (30 μg). Each colony was plated on to a DB agar plate so the whole plate was covered with cells, which creates a lawn when incubated. Then the 6 antibiotic disks were placed onto the plate in a circular fashion and incubated for 48 hours at room temperature. The zone of inhibition was recorded based on the measurement of lack of growth around the disk.

There were 26 colonies that were resistant to seven or more antibiotics in the previous two experiments, and were chosen for further investigation. In order to figure out the concentration range in which these bacterial colonies are susceptible or resistant to the antibiotics, a 96 well plate was used. A stock solution of 0.04g of antibiotic and 10mL of sterile water was created. This was then distributed into 8 test tubes filled with TSB in increasing order of concentration. The concentrations determined were in terms of μg/mL. The ratios used in this experiment were as follows: 64, 32, 16, 8, 4, 2, 1, ½ (and sometimes ¼). The amount of antibiotic added to each 15 mL test tube of TSB is: 64-240 μl, 32-120 μl, 16-60 μl, 8-30 μl, 4-15 μl, 2-7.5 μl, 1-3.8 μl, and ½- 1.9 μl (with ¼ being 1 μl). These concentrations are then pipetted into a standard 96 well plate in order of increasing concentration. Each of the highest concentrations for each corresponding antibiotic are listed here: penicillin G.- 5.52 mM, erythromycin- 5.03 mM, ampicillin- 10.57 mM, amoxicillin- 10.11 mM, nalidixic acid- 15.9 mM, neomycin sulfate- 5.18 mM, streptomycin sulfate- 6.35 mM, and tetracylcine- 8.31 mM. After these well plates had incubated for 48 hours at room temperature, each plate was put through an optical density reader. These numbers were recorded and analyzed in further detail to figure out resistance level of each of these 26 organisms. A control was also used in order to ensure accuracy of tests. The control was *Corynebacterium renale* and is very susceptible to antibiotic treatments; meaning tests were successful when this bacterium was susceptible to any level of antibiotics.

In order to obtain a resistance coefficient to quantify overall resistance of a bacterium to a particular antibiotic, the optical density values that were recorded needed to be analyzed. Each "well" had to be adjusted for the optical density values of the TSB and the differing antibiotic concentrations. The optical density values of each column in the well plate containing the same concentration of antibiotic with bacteria, were subtracted from the corresponding optical density recording of the same concentration of antibiotic without bacteria. This allows for the readings to then only contain the growth of the cells instead of the growth of cells, TSB, and antibiotic. The optical density of a specific concentration of antibiotic and isolate was divided by the corresponding well that contained only the isolate and TSB. This coefficient then represents the amount of growth of the isolate in the well with antibiotic compared to normal growth of the isolate in regular conditions (TSB). These coefficients were then graphed with the resistance coefficient on the y-axis and the organism isolate on the x-axis. The eight different bars for each organism represent the eight different antibiotic concentrations and the corresponding resistance to that concentration. All graphs can be seen in the appendix.

Once the result of each colony on each antibiotic was recorded, this information was taken and these antibiotics were then combined. The three combinations of antibiotics tested were: penicillin G. and streptomycin sulfate, penicillin G. and nalidixic acid, and streptomycin sulfate and kanamycin sulfate. These combinations were created based on the mechanisms used to affect the bacteria. Streptomycin sulfate and kanamycin sulfate act against protein synthesis while nalidixic acid works against DNA formation and penicillin G. works against the cell wall. Therefore, by combining different classes of drugs and analyzing resistance, it gives a better picture of what resistance mechanisms might be present in the organism. For each combination of antibiotics, 0.4g of each antibiotic was added to 600 mL of DB media after it had been autoclaved and brought down to 60˚C. The concentration of the antibiotic plates was calculated by taking 0.4g and dividing it by the volume of DB, which was 0.6L. Then that number was divided by the molecular weight of the antibiotic and then multiplied by 1000 to get mM. All of these concentrations were kept much lower than clinical dosage to gain an effective measure of baseline resistance levels of each organism.

The antibiotic plates created initially had very high concentrations relative to the standard peak serum levels commonly seen in patients given normal antibiotic dosages. These plates were used as a baseline measure to see levels of resistance to very high concentrations and do further analysis on specific isolates of interest. If the isolates grew on high concentration plates of antibiotics, then it is appropriate to test it against increasing concentrations of the antibiotic to see its overall resistance level. The antibiotic disks were used to see the level of resistance by measuring the zone of inhibition. The larger the zone of inhibition, the more powerful the antibiotic is on that particular bacterium.

Identifying Samples:

After tests had been run, the 26 organisms of interest were identified using standard microbiological tests along with running PCR sequencing. The following tests were run in order to identify these organisms: glucose fermentation and nitrate reduction broths, EMB and TSA plates, TSI slant, Citrate slant, Gelatin deep, and SIM deep. Both glucose fermentation and nitrate reduction were done by inoculation of the tubes with a loop-full of the culture using a small portion of the colony and then incubating at 37° C. the glucose fermentation was checked after 20 hours and the nitrate reduction was checked after 72 hours. The TSI slant was done by inoculating the tube by streaking the slant then stabbing the bottom of the tube, leaving the cap loose and incubating at 37°C for 20 hours. SIM deep and gelatin deep are both inoculated by stabbing the agar/gelatin with a straight inoculating needle at 37°C for 72 hours. The EMB plates and TSA plates are both streaked out of isolation and incubated at 37° C for 20 hours (13).

For the polymerase chain reaction (PCR), DNA was extracted using the Zymo Research Fungal/Bacterial DNA MiniPrep kit. To start off, 200 μl of sample was put into a microcentrifuge tube and centrifuge for 1 minute or until pellet of cells forms on bottom. Procedures can be found in ZR Fungal/Bacterial DNA MiniPrep Instruction Manual (12).

Once the DNA was extracted, these samples were taken to the nanodrop machine to verify the amount of DNA present. Once this was verified, PCR was initiated. The cycles are as follows: 94 °C for 30 seconds, 55°C for 1 minute, and 72°C for 1 minute 35 times. The sample is then brought down to 4°C. These PCR products are then subject to gel electrophoresis with a 1% agarose gel in order to verify successful PCR (14).

Results

Each strain of bacteria that was collected for analysis was resistant to at least one antibiotic. The breakdown of the 26 isolates chosen for further study in regards to number of antibiotics in plate form that they were resistant to are as follows: one isolate resistant to 1-3 antibiotics, 11 isolates resistant to 4-6 antibiotics, and 14 isolates resistant to 7-8 antibiotics. These eight antibiotics were done in 96 well plates. As for the distribution of the 125 isolates as a whole in terms of number of antibiotics they are resistant to are as follows: 71 isolates resistant to 0-4 antibiotics, 18 isolates resistant to 5-6 antibiotics, 18 isolates resistant to 7-9 antibiotics, and 18 isolates resistant to 10-14 antibiotics. The total of 14 antibiotics used came from the 8 antibiotics in plate form and 6 antibiotics in disc form. Overall, there were 43.2% of all 125 isolates tested were resistant to five or more antibiotics. All isolates were capable of growing in the presence of at least one antibiotic. This data is representative of the preliminary data gathered on these isolates. The concentrations of the plates in mM are: streptomycin sulfate- [.01146]mM, ampicillin-[.191]mM, nalidixic acid-[.287]mM, penicillin G.-[.199]mM, erythromycin-[.0908]mM, neomycin sulfate-[.108]mM, vancomycin-[.0460]mM, and tetracycline-[.150]mM. In Table 1 below are the common dosage amounts that are normally given out in a clinical setting along with peak serum concentration as well in order to quantify how much of the antibiotic gets into the blood stream. In addition, the peak serum concentrations have been converted to mM in order to make comparisons easier with tests done during the experiment.

Table 1: Table consisting of the clinical dosage and peak serum concentration of each antibiotic and the corresponding converted concentration.

Antibiotic	Common Dosage	Peak Serum Concentration	Concentration (mM)
Amoxicillin	250 mg per 8 hours	5 μg/mL	[0.0137] mM
Ampicillin	250-500 mg per 6 hours	3 μg/mL	[.00859] mM
Streptomycin Sulfate	1g a day	25-50 μg/mL	[.08596] mM
Nalidixic Acid	4g per day	20-40 μg/mL	[1.7226] mM
Penicillin G.	1 million units	14.4 μg/mL	[.04306] mM
Erythromycin	250 or 500 mg 4 times a day	7.21 μg/mL	[.00982] mM
Neomycin Sulfate	4g a day	6.1 μg/mL	[.00993] mM
Vancomycin	1g per 12 hours	20 μg/mL	[.0138] mM
Tetracycline	125 to 500mg per 12 hours	4-5 μg/mL	[.01125] mM

The concentration of the initial DB agar plates with antibiotic were then calculated based on a percentage level of the peak serum concentration in order to gain knowledge on the level of resistance these bacteria have. These percentages are as follows: streptomycin sulfate-13.33%, ampicillin-2223.5%, nalidixic acid-16.66%, penicillin G.-462.15%, erythromycin-924.64%, neomycin sulfate-1087.61%, vancomycin-294.2%, tetracycline-1333.33%. The total breakdown can be seen in Table 2. The concentrations of the plates prove to be high concentrations based on the percentage of the peak serum levels normally found in patients after being given normal dosages.

Table 2: Concentration of antibiotics in DB plates represented as a percentage of the peak serum concentrations found in patients.

Antibiotic	Concentration of Plates	Peak Serum Concentration	% of Peak Serum Concentration
Streptomycin Sulfate	0.01146 mM	[0.08596] mM	13.33%
Ampicillin	0.191 mM	[.00859] mM	2223.5%
Nalidixic Acid	.287 mM	[1.7226] mM	16.66%
Penicillin G.	.199 mM	[.04306] mM	462.15%
Erythromycin	.0908 mM	[.00982] mM	924.64%
Neomycin Sulfate	.108 mM	[.00993] mM	1087.61%
Vancomycin	.0406 mM	[.0138] mM	294.2%
Tetracycline	.150 mM	[.01125] mM	1333.33%

To further investigate, 26 isolates of the most resistant bacteria strains were put into 96 well plates allowed to grow and then read using an optical density reader as mentioned previously. The optical densities were then converted into resistance coefficients, which measured individual levels of resistance for each isolate at each concentration of antibiotic (5). There were eight concentrations tested for each of the seven antibiotics. Graphical representations of each of the 26 isolates against the eight antibiotics can be found in the Appendix. The number of isolates resistant to the highest concentration of antibiotic (and ultimately all concentrations leading up to this one) differs between antibiotics. The results can be found in Table 3.

Table 3: Isolate resistance to highest concentration of different antibiotics and the corresponding percentages

Antibiotic	# of Isolates Resistant to Highest Concentration	Percent of Isolates Resistant to Highest Concentration
Erythromycin	21	80.80%
Amoxicillin	20	76.92%
Ampicillin	21	80.80%
Streptomycin Sulfate	21	80.80%
Tetracycline	0	0%
Neomycin Sulfate	15	57.70%
Nalidixic Acid	22	84.60%

This high concentration was analyzed by taking the peak serum concentration as a percent of the highest concentration. The results of this calculation indicate the percentages to be: erythromycin- 0.195% of highest concentration, penicillin G.-0.390%, amoxicillin-0.136%, ampicillin-0.081%, streptomycin sulfate-1.35%, tetracylcline-0.135%, neomycin sulfate-0.192% and nalidixic acid-10.83%. Knowing these isolates are very resistant to concentrations much higher than peak serum concentrations led to another experiment to further analyze resistance levels.

Initially, DB plates with both streptomycin and penicillin G. were created to measure baseline resistance levels with high concentrations. Eight out of the 26 isolates were resistant to both [0.0687] mM of streptomycin sulfate and to [0.196] mM of penicillin G. This led to combinations of these antibiotics at even higher concentrations to measure their overall resistance levels. The combined treatment of the antibiotics streptomycin sulfate and penicillin G. was used from a stock concentration of 0.4g of each antibiotic in 10mL of sterile water. This stock concentration was then diluted into eight different concentrations as done previously for the 96 well plates. Using TSB as the media, the eight different concentrations for streptomycin sulfate and penicillin G. can be seen in Table 4. Below are graphical representations of each isolate chosen to test against this combination in each antibiotic separately to show initial resistance levels.

Table 4: Final concentrations for streptomycin sulfate and penicillin G. after addition to TSB media. These concentrations were added together in the 96 well plate to create a combination antibiotic treatment.

Concentration (mg/mL)	Streptomycin Sulfate	Penicillin G.
[64]	63.5 mM	110.42 mM
[32]	31.7 mM	55.2 mM
[16]	15.9 mM	27.6 mM
[8]	7.94 mM	13.8 mM
[4]	3.97 mM	6.9 mM
[2]	1.98 mM	3.45 mM
[1]	1.01 mM	1.75 mM
[1/2]	0.503 mM	0.875 mM

The final outcomes of the optical density values from the 96 well plates were then analyzed based on resistance coefficients explained in the method section. These values were then assembled into the graph seen in Fig. 5. Figures 1-4 are showing the fact that each of these organisms in Figure 5 were in fact resistant to both streptomycin sulfate and penicillin G. on their own. They all showed resistance coefficients of 0.8 or higher for every level and are therefore able to be used for the experiment in Figure 5.

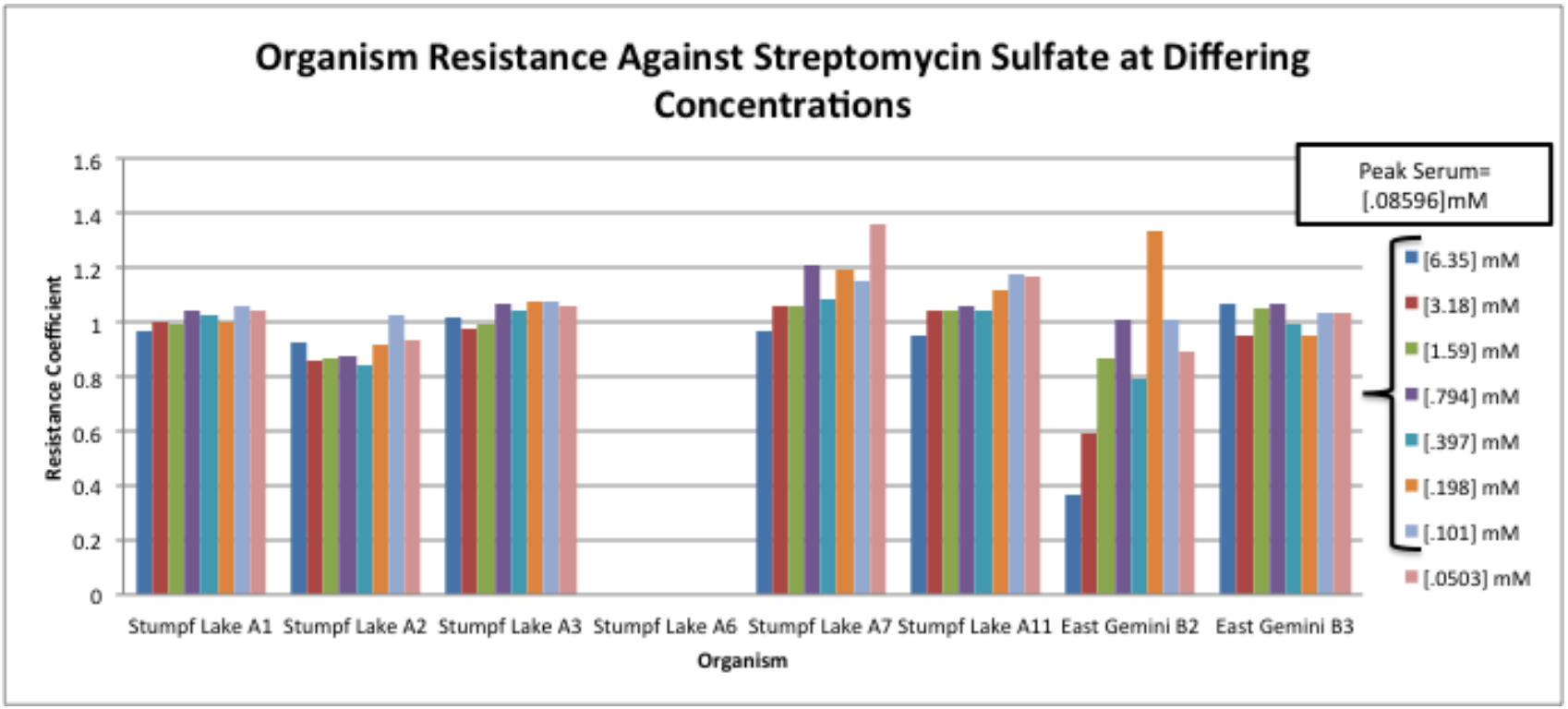

Figure 1: Resistance Coefficients for organism A1-B3 in differing concentrations of streptomycin sulfate. Bracket indicates levels above peak serum concentration.

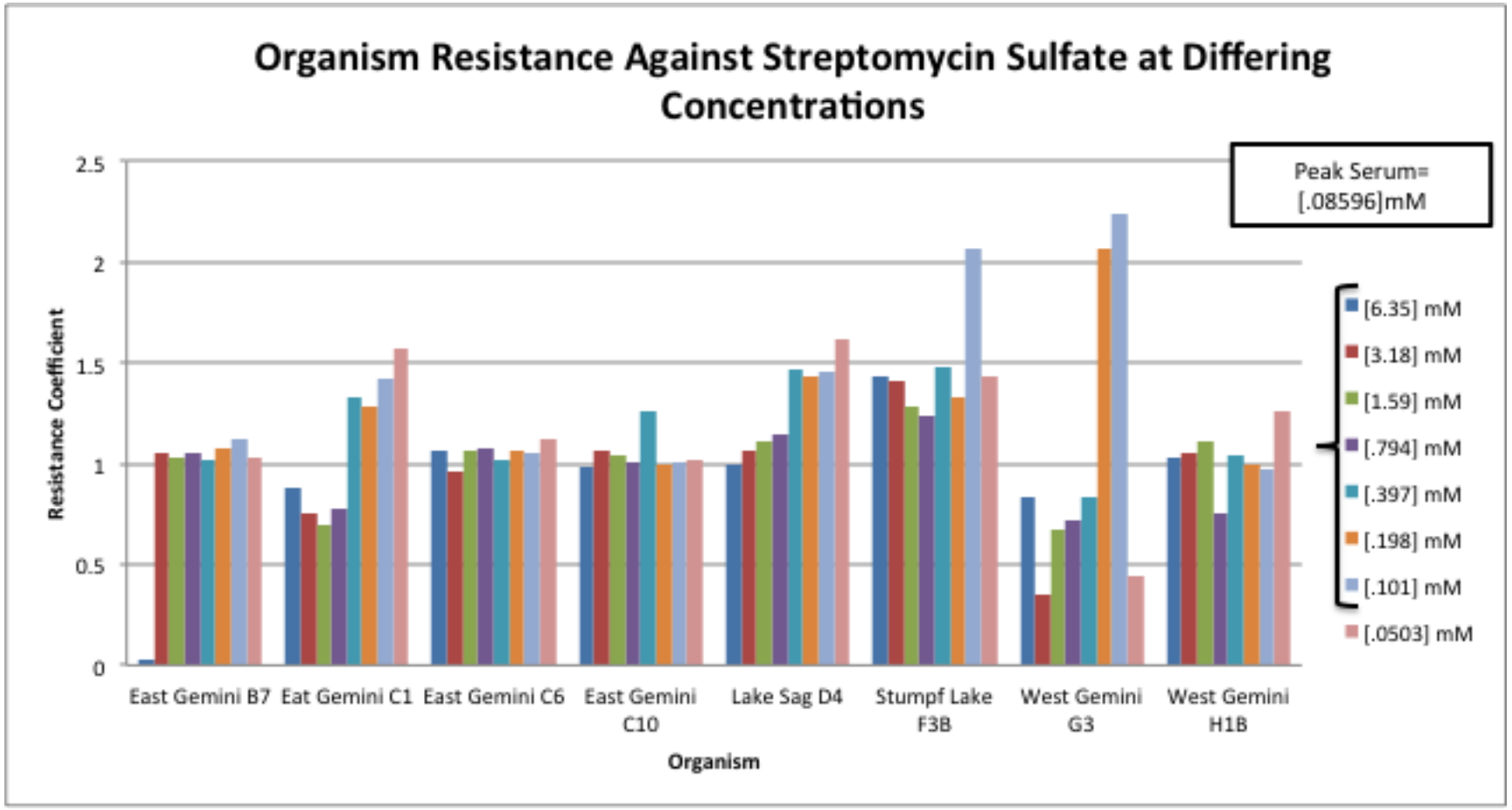

Figure 2: Resistance Coefficients for organism B7-H1B in differing concentrations of streptomycin sulfate. Bracket indicates levels above peak serum concentration.

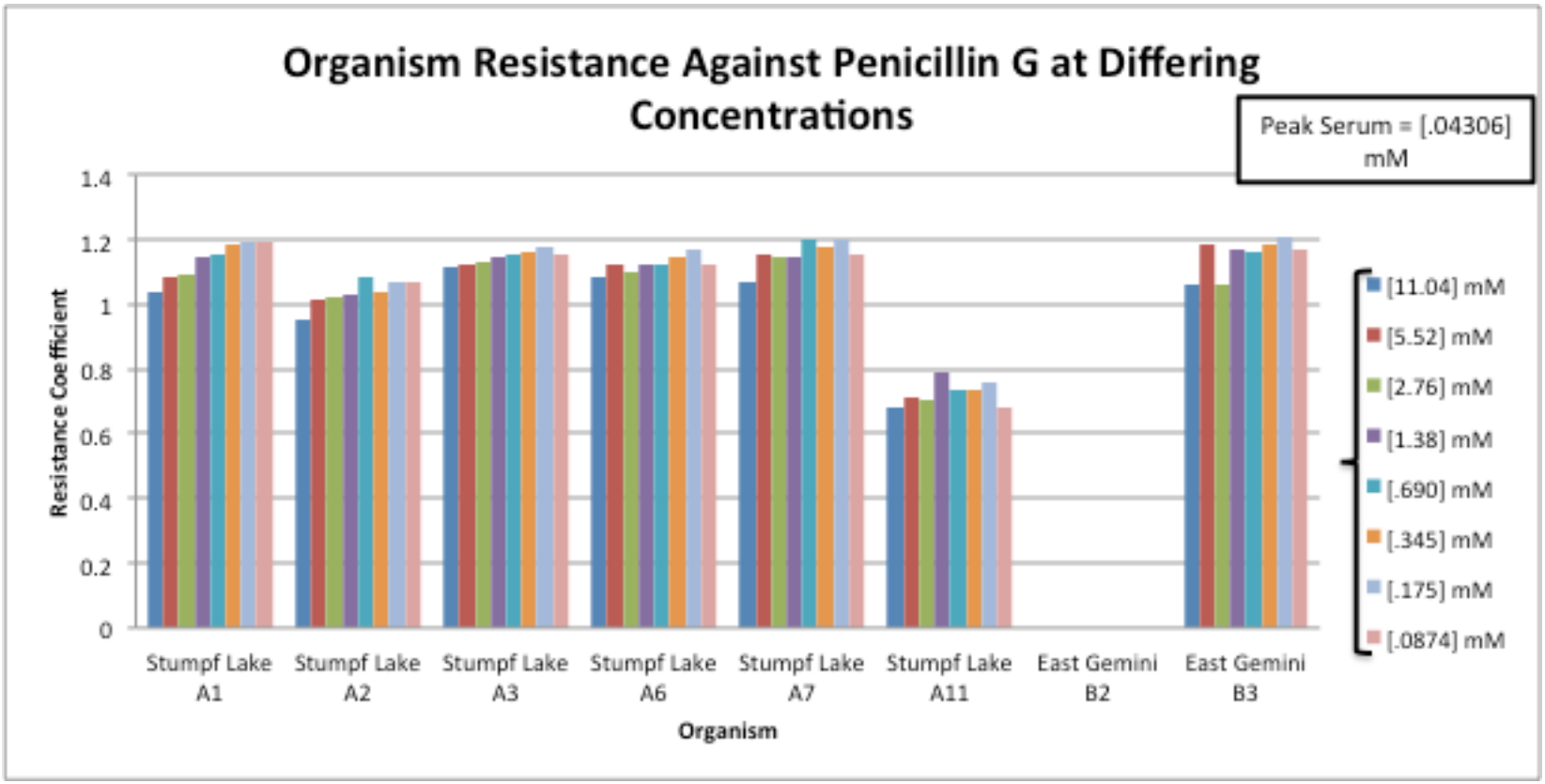

Figure 3: Resistance coefficients for organisms A1-B3 in differing concentrations of penicillin G. Bracket indicates levels above peak serum concentration.

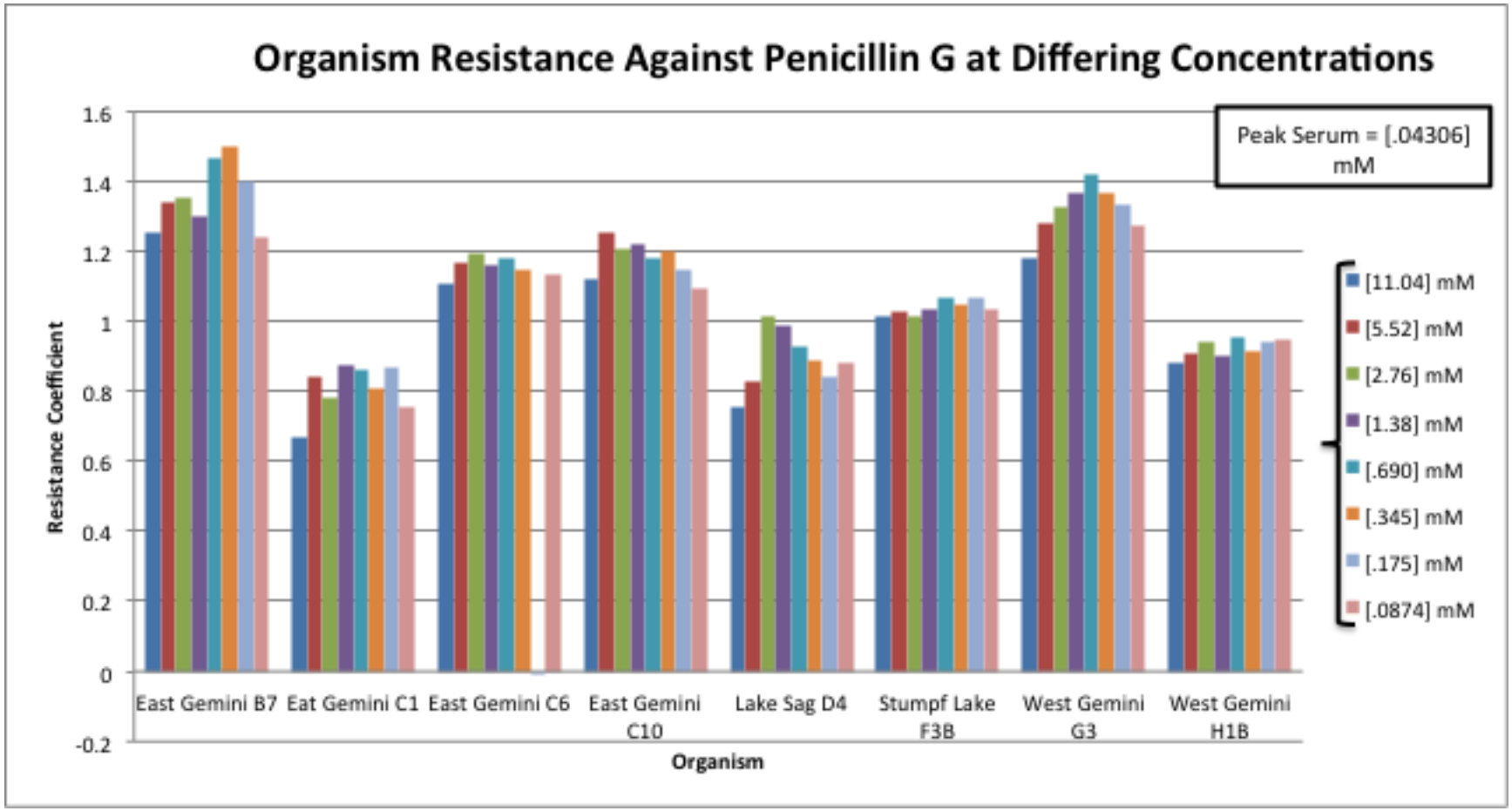

Figure 4: Resistance coefficients for organisms B7-H1B in differing concentrations of penicillin G. Bracket indicates levels above peak serum concentration.

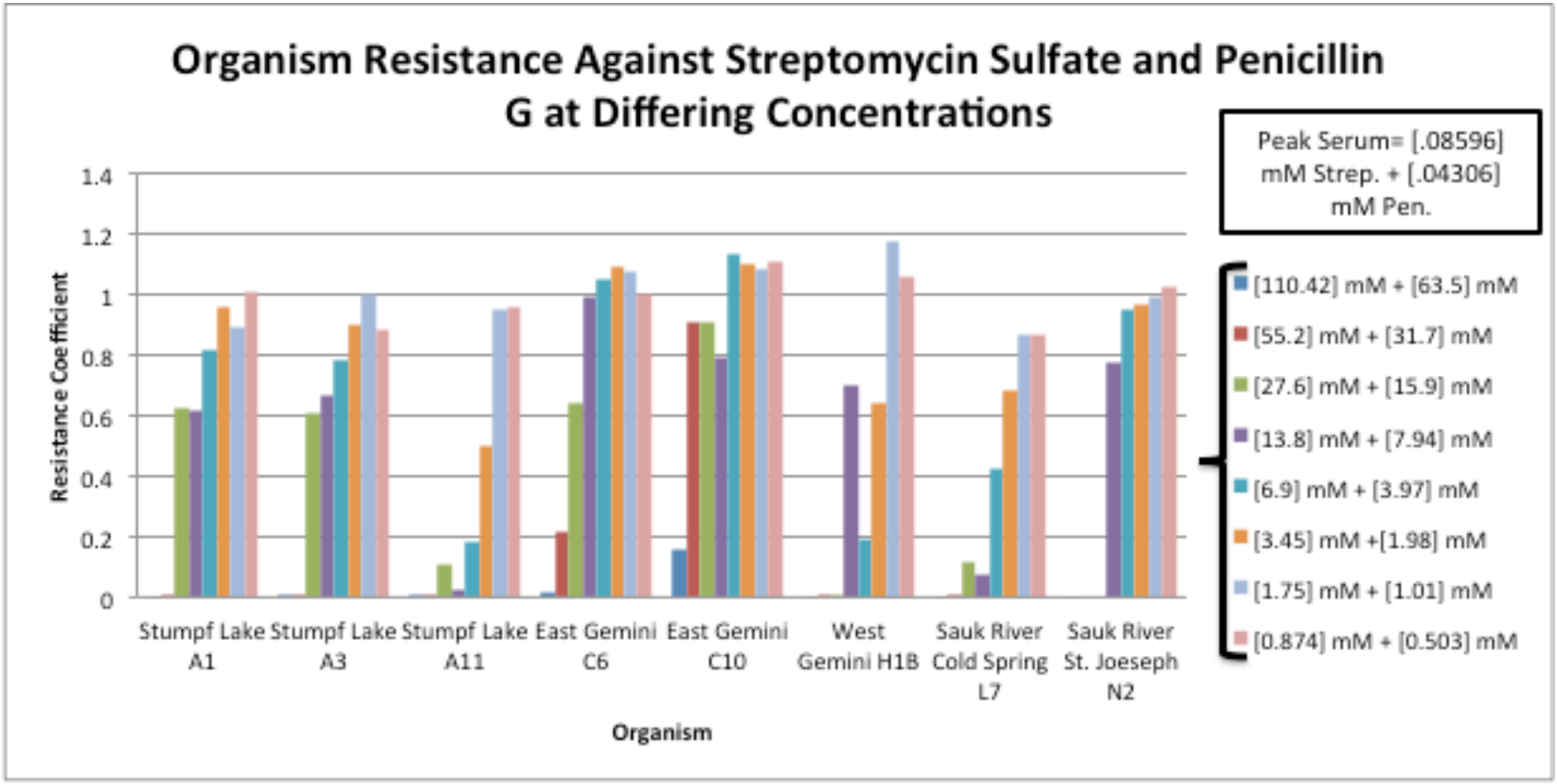

Figure 5: Graph representation of the effectiveness of streptomycin sulfate and penicillin G. combined at differing concentrations. Resistance levels in the bracketed area represent resistance to concentrations higher than peak serum concentration for each antibiotic. Sample A is from Stumpf Lake, sample C is from East Gemini Lake, sample H is from West Gemini Lake, Sample L is from a river downstream of farmland in Cold Spring, MN and sample N is from the Sauk River in St. Joseph, MN near a Frisbee golf course

Figure 5 shows that everything is higher than the peak serum concentration. All isolates were resistant to at least two concentrations higher than the peak serum concentration. Four out of the eight tested are resistant to three or more of the concentrations above peak serum levels.

The results of the different standard microbial tests revealed the genera of each isolate. The two most common genera were Acinetobacter and Flavobacteria. For Acinetobacter, seven out of 26 were classified under this genus. Through analyzing the antibiotic plates and the 96 well plates, this genus is highly resistant to both the protein synthesis disruption antibiotics and cell wall disruption antibiotics. The cell wall disruption antibiotics includes: penicillin G., ampicillin, amoxicillin, and vancomycin. The protein synthesis disruption antibiotics includes: neomycin sulfate, streptomycin sulfate, tetracycline, and erythromycin. The concentration of resistance for each isolate in this genus for the corresponding antibiotic class can be seen in Table 5. The numbers reported show a high resistance level to both classes of antibiotics. Tetracycline, which is a protein synthesis disruption antibiotic, had no growth on the plates and very little growth in the 96 well plates, which explains the 75% resistance levels in the Table 5. The reason resistance levels to tetracycline were low are unknown.

Table 5: Isolates under the genera Acinetobacter and their corresponding level of resistance to different classes of antibiotics. Within each class of antibiotic, four antibiotics were used. Percentages were calculated based on total amount of resistance over total antibiotics used.

Acinetobacter Isolate	Cell Wall Disruption Antibiotics	Protein Synthesis Disruption Antibiotics
A1	4 out of 4	3 out of 4
B7	4 out of 4	3 out of 4
C10	4 out of 4	3 out of 4
F3B	4 out of 4	3 out of 4
H3B	1 out of 4	3 out of 4
L6	4 out of 4	3 out of 4
N2	3 out of 4	3 out of 4
Resistance Percentage	89.30%	75%

The other common genus of bacteria present in these samples was Flavobacteria. Eight out of the 26 isolates chosen fell into the genera. Again, through analyzing the antibiotic plate growth and the growth in the 96 well plates, high levels of resistance were found. The amount of resistance of each isolate to each antibiotic class can be found in Table 6.

Table 6: Isolates under the genera Flavobacteria and their corresponding level of resistance to different classes of antibiotics. Within each class of antibiotic, four antibiotics were used.

Flavobacteria Isolate	Cell Wall Disruption Antibiotics	Protein Synthesis Disruption Antibiotics
A2	4 out of 4	3 out of 4
A3	4 out of 4	3 out of 4
A6	4 out of 4	2 out of 4
A7	4 out of 4	3 out of 4
A11	4 out of 4	3 out of 4
C1	4 out of 4	3 out of 4
C6	4 out of 4	3 out of 4
G3	3 out of 4	3 out of 4
Resistance Percentage	96.90%	71.90%

The other genera represented in these 26 isolates were Enterobacter with five isolates, Pseudomonas aeruginosa with two isolates, Proteus with one isolate, Klebsiella with one isolate, and Salmonella with one isolate.

After the genera were sorted out, it was important to look at the different classes and how many isolates were resistant to each class. It can be seen that of the 26 isolates, 88% were resistant to the cell wall disruption antibiotics, 81% were resistant to protein synthesis disruption, 67% were resistant to cell membrane disruption antibiotics, and 85% were resistant to DNA disruption antibiotics.

In addition, figure 6 shows the different locations that each of these 26 resistant isolates were found.

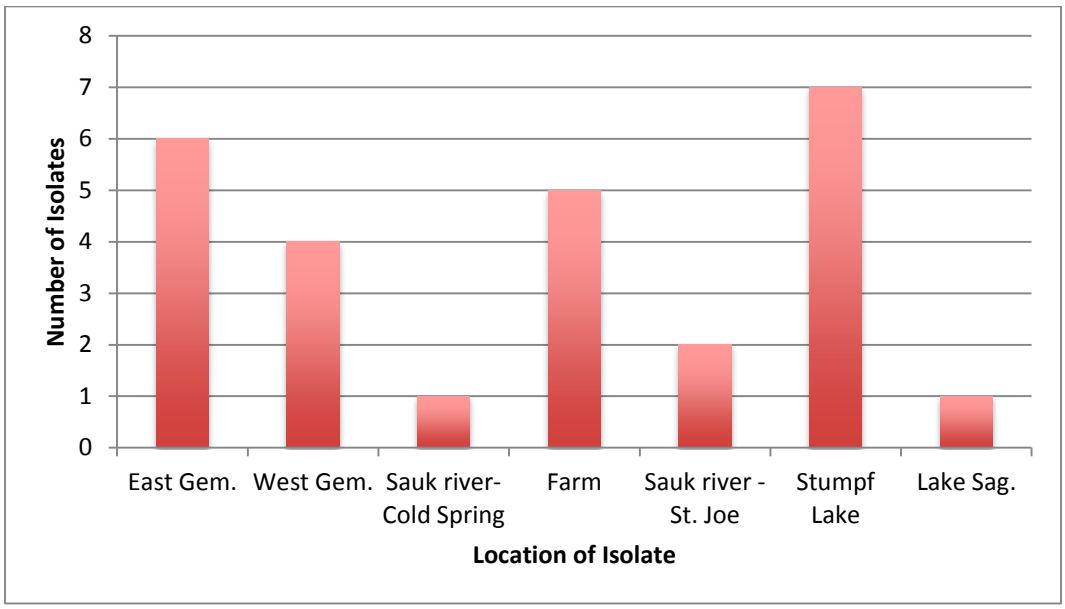

Figure 6: Breakdown of the locations of the 26 highly resistant isolates.

Discussion/Conclusion:

The studied isolates from the lakes and streams in central Minnesota did in fact show high levels of resistance to several antibiotics in different antibiotic classes. When tested in the 96 well plates, these isolates were able to grow as well as, if not better, in high concentrations of antibiotic (some concentrations above the normal clinical amount) as measured by the high values of the calculated resistance coefficient. The bacteria were deemed resistant if the coefficient was 0.8 or above. An interesting situation arises when Table 2 and Table 3 are analyzed further. With the 26 isolates chosen to study further, five of the eight antibiotics had an 80% or higher level of resistance to the highest concentration given according to Table 3. This is important when looking at the percentage of the highest concentration in terms of peak serum concentrations in patients as recorded in Table 2. Of the five antibiotics that had 80% or higher resistance, most of them have a peak serum percent that is lower than 1% of the highest concentration, with two of them lower than 11%. That means these isolates are able to withstand roughly 80 times higher than the peak serum level normally found in patients given average dosage amounts. As stated earlier, the ability for environmental bacteria to transfer resistance to pathogenic bacteria is not an uncommon occurrence. If these bacteria are able to transfer genes for resistance against commonly used medical antibiotics, there could be potentially serious trouble in treating future infections. These isolates were found in lakes and streams of rural areas, not in direct contact with medical facilities. Therefore, the levels of resistance should not be high, unless there is substantial antibiotic contamination from humans, farms, or factories further away. This would lead to the thought that rivers, like the ones that were tested, could be a

reservoir for resistance genes. There were many different types of bacteria that were studied and since almost all of them had similar resistance mechanisms, it can be hypothesized that these isolates were able to receive resistance genes from other aquatic bacteria. Since there is no direct input of antibiotic contamination/waste, the overall concentration of each of the antibiotics within these studied bodies of water is hypothesized to be extremely low.

In addition, when looking closer at the two most common genera of bacteria, there are also important things to note. Acinetobacter, which is a genera of bacteria commonly found on the skin, was the second most prevalent bacteria in the 26 studied isolates. Broken down in Table 5, it can be seen that these isolates were very resistant. Given the isolate and the two classes of antibiotics commonly used, cell wall disruption and protein synthesis disruption, it can be noted how resistant these isolates are. There was 89.3% resistance to cell wall disruption and 75% resistance for protein synthesis disruption. Each of these classes had four different antibiotics to gage level of resistance. The reason there is only 75% resistance for protein synthesis disruption is because none of the isolates were able to grow on tetracycline. This is an odd finding because tetracycline is commonly found in animal feed and therefore commonly found in soil fertilizer because of the use of manure. Regardless of this fact, these isolates are very resistant to two of the first line classes of antibiotics given to patients. This gives an inclination to think these bacterial isolates may have multiple mechanisms of resistance in action in order to be able to be highly resistant to multiple classes of antibiotics. Even though the isolates tested are not themselves pathogenic, they have the potential to transfer their resistance genes to pathogenic bacteria that are resistant to a high percentage of commonly used antibiotics. Another possibility may just be the porins that are common to gram-negative bacterial isolates are filtering out the antibiotics, and therefore, not allowing them to attack the isolates. The same analysis can be done on the most prevalent bacteria genera of the 26 studied, Flavobacteria. This genus is commonly found in the aquatic ecosystem and has been known to cause disease in rainbow trout. This data is compiled in Table 6 and states that 96.9% of these Flavobacteria isolates were resistant to cell wall disruption antibiotics, and 71.9% were resistant to protein synthesis disruption antibiotics. These numbers also prove to be high and pose similar problems as Acinetobacter isolates do. Instead of directly effecting human pathogens, this may have a huge impact on the wellbeing of the aquatic animals in these ecosystems if these resistance mechanisms are transferred to aquatic pathogens. In the future, this could cause for a disruption in the ecosystem if it is left the way it is now.

When looking at these isolates in terms of their resistance levels to different classes of antibiotics, the number is relatively high. In the results section, the individual class resistance levels that were shown related to the 26 isolates. Analyzing these numbers closer, it can be seen that 57.7% of the 26 isolates were resistant to all four classes of antibiotics tested in this experiment. It is important to note the point that over half of the isolates tested had resistance for four different kinds of antibiotic attacks. This goes along with the idea of a great amount of gene transfer between bacterial genera. It also poses the question of what initial resistance

mechanisms do these bacteria have, and what caused them to expand their mechanisms given their environment? The issue that makes this high percentage important is the fact that as a society there are limitations to the number of effective antibiotics and antibiotic classes. Having these 26 isolates be highly resistant to four of the main classes of antibiotics is a cause for great concern.

In addition, it is important to look at where these highly resistant isolates were located relative to each other. Table 6 shows the location with the most isolates that were highly resistant were Stumpf Lake and East Gemini Lake. East Gemini Lake is where the wastewater treatment is located, which proves to be a very interesting finding. In addition, Stumpf Lake may have pressure from agricultural farm run-off based on its location. However, the most interesting thing to look at is the fact that Lake Sagatagan only has one of the highly resistant isolates. This lake is commonly used for swimming and other water activities meaning if the resistance was due to human contamination, this location should have a lot more highly resistant isolates. Also, throughout the experiments, there were no isolates of either *Escherichia coli* or *Enterobacter.* Both of these genera of bacteria would give rise to the notion of fecal contamination due to humans. However, since there were no isolates found in either of these genera, it is interesting to note that human use of antibiotics may not be the pressure creating these highly resistant bacteria in these aquatic environments.

In attempts to be able to destroy the pathogens that show antibiotic resistance, physicians may try to prescribe antibiotics in combinations, whether it be of the same class or taking two different classes. An experiment was done by looking at streptomycin sulfate of the protein disruption class in combination with penicillin G. of the cell wall disruption class. These antibiotics were used in abnormally high concentrations in order to determine at which concentration the isolates would be killed. What was found can be seen in Figure 5. This graph shows that although these isolates were not resistant to all of the increasing concentrations, they were resistant to concentrations higher than the peak serum concentration for both antibiotics. Every isolate is resistant to at least two steps above peak serum concentration with this combination scenario. Four of the eight isolates tested are resistant to at least three levels above the peak serum concentration. Even though this is a small sample size, it is interesting to see this many isolates resistant to these abnormally high concentrations of antibiotics when they are in the aquatic environments of small towns and college campuses, not subjected to direct antibiotic stress.

The fact that the 26 aquatic bacteria that were tested in depth were resistant to seven or more antibiotics at high concentrations is an issue for treatment of infections for humans or aquatic animals living in these environments. The 125 isolates collected showed a high level of resistance to these common antibiotics as well. Aquatic bacteria have the ability to form reservoirs of resistance mechanisms where there is the ability to transfer mechanisms to different communities of bacteria. This is a danger to both humans and aquatic animals because there is potential for pathogenic bacteria to be able to gain these resistance mechanisms from these

locations. In addition, there is a high potential for treatment of infections consisting of these bacteria to be far more difficult in the future. Great concern should be taken not only with pathogenic, clinically relevant bacteria, but also with the amount of resistance in the natural ecosystem that can serve as a harbor of resistance for these pathogens.

References:

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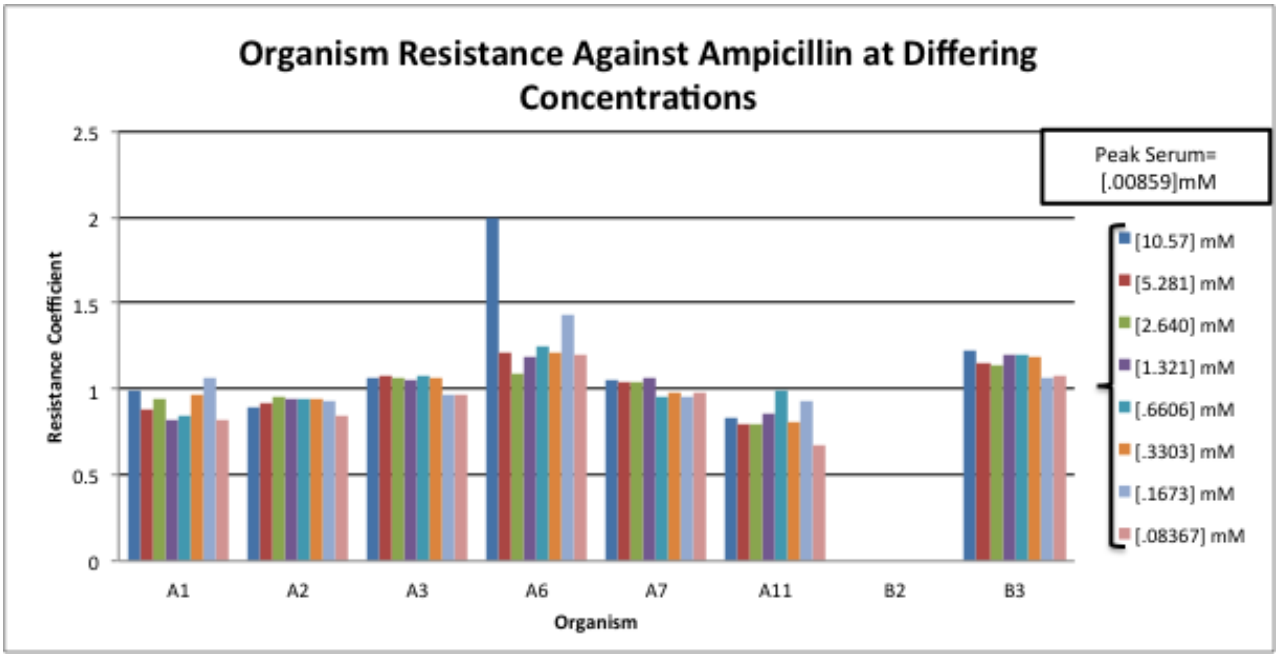
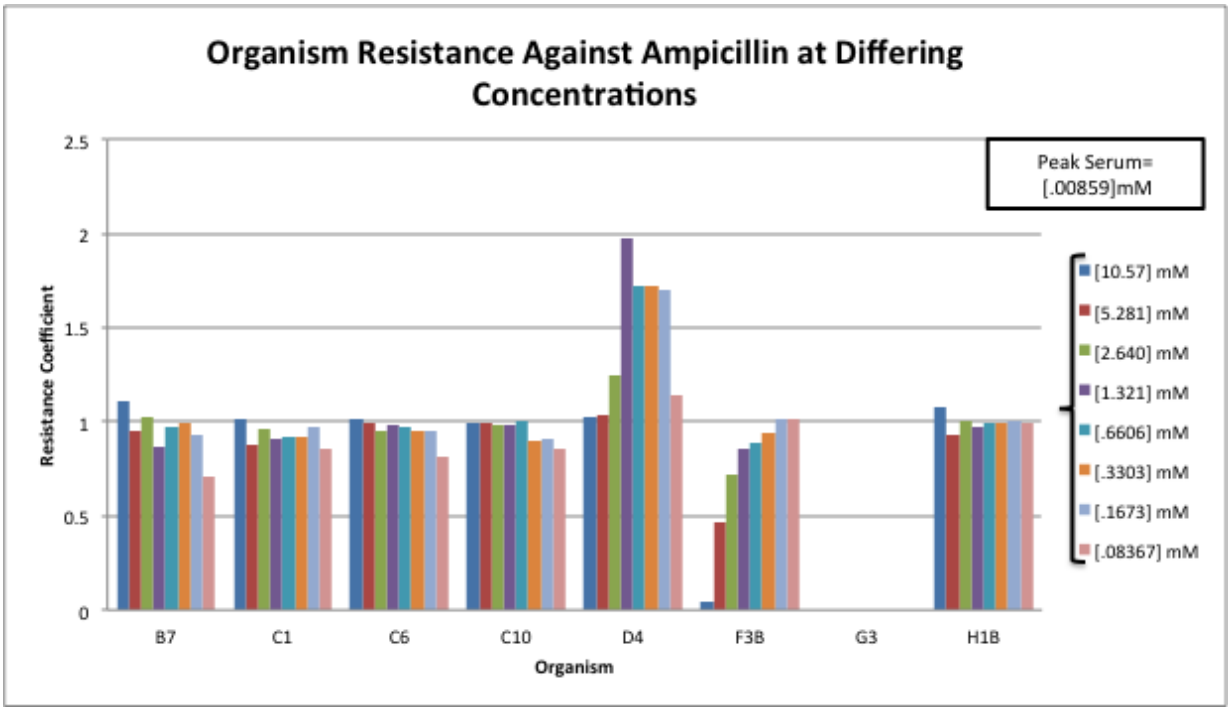
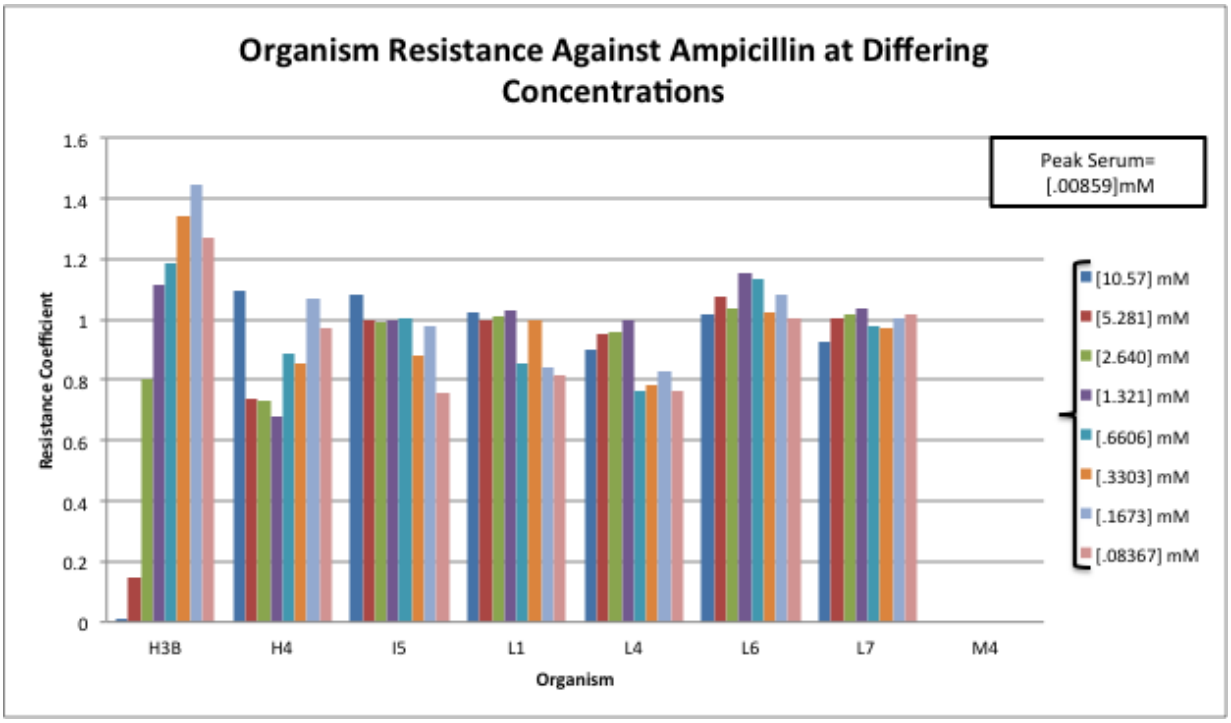
Appendix

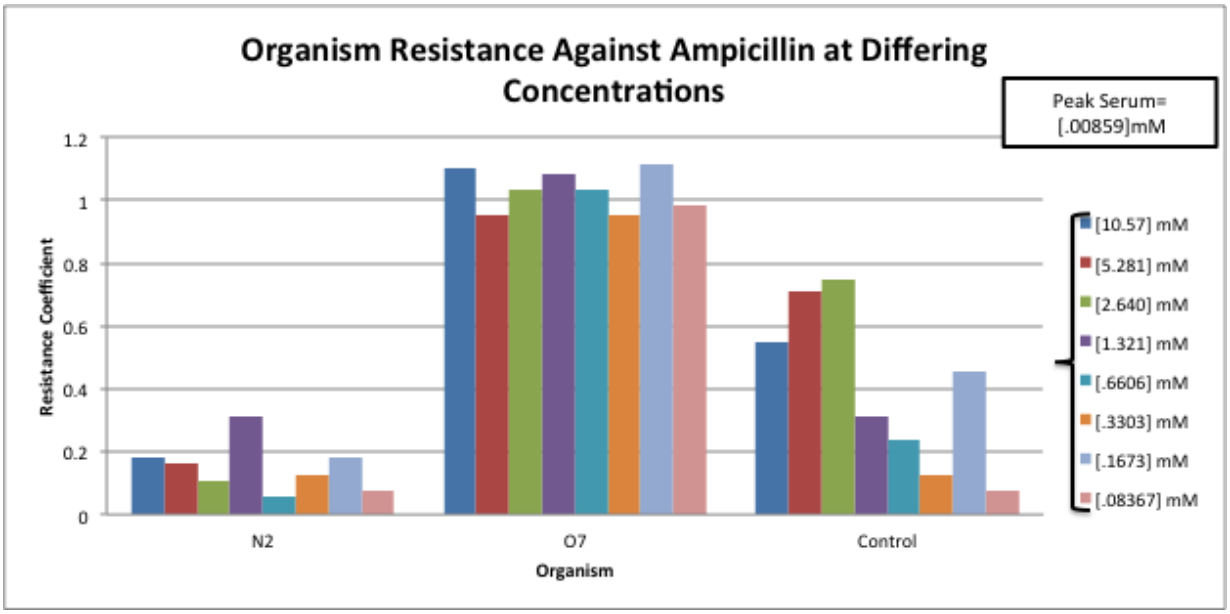
The following are the rest of the graphs constructed with each isolate under each antibiotic including: Ampicillin, Amoxicillin, Erythromycin, Nalidixic Acid, Neomycin Sulfate, Penicillin G., Streptomycin Sulfate, Tetracylcine. Isolates are named by letter instead of location (ie A, B, C). The complete breakdown of where each isolate came from can be found on page 5.

Ampicillin:

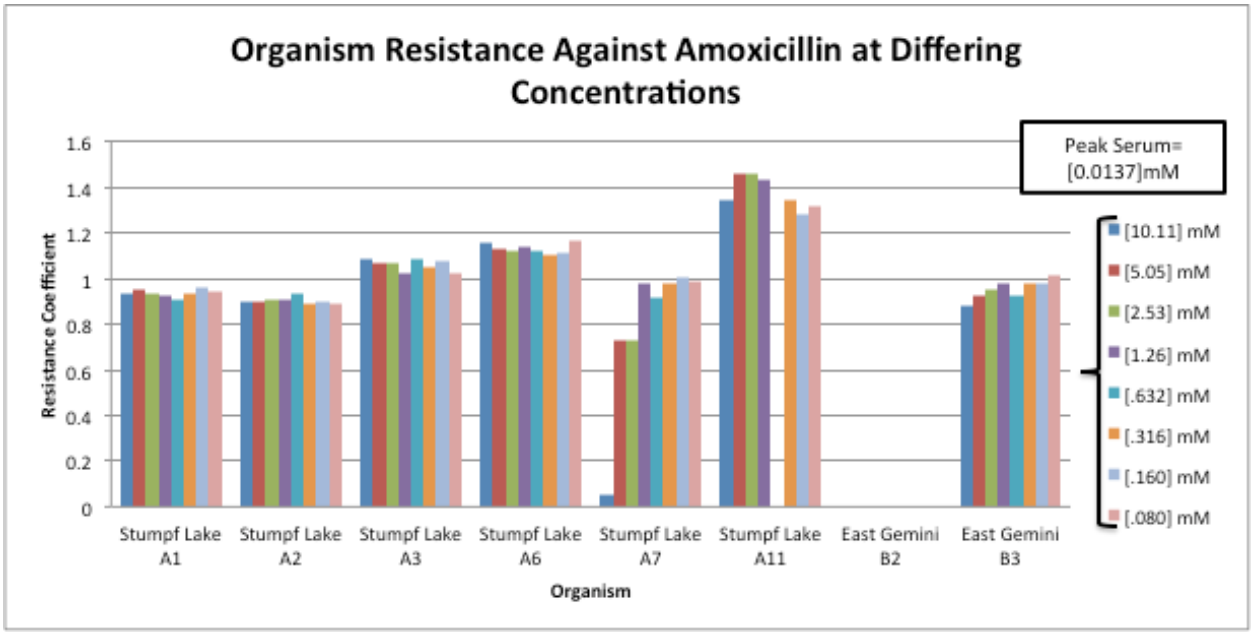


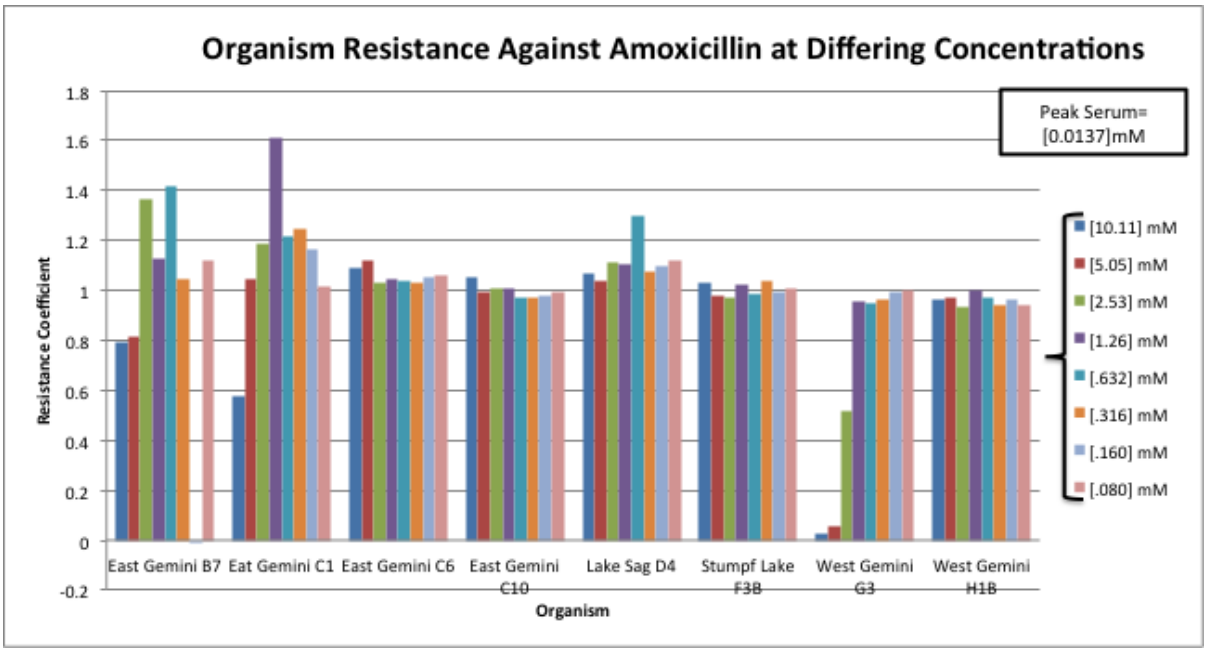

Organism Resistance Against Ampicillin at Differing Concentrations
Peak Serum= [.00859]mM
Resistance Coefficient
2.5
2
1.5
1
0.5
0
B7
C1
C6
C10
D4
F3B
G3
H1B
Organism
[10.57] mM
[5.281] mM
[2.640] mM
[1.321] mM
[.6606] mM
[.3303] mM
[.1673] mM
[.08367] mM

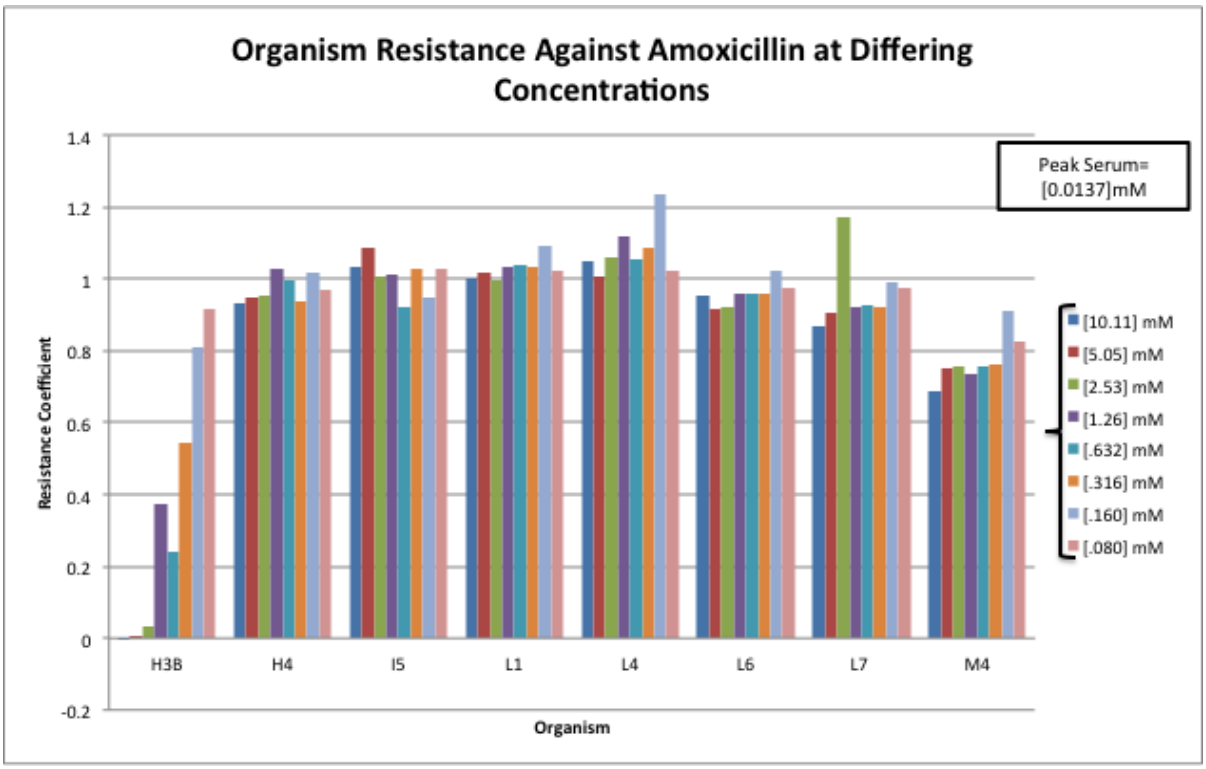

Organism Resistance Against Ampicillin at Differing Concentrations
Peak Serum= [.00859]mM
Resistance Coefficient
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
H3B
H4
I5
L1
L4
L6
L7
M4
Organism
[10.57] mM
[5.281] mM
[2.640] mM
[1.321] mM
[.6606] mM
[.3303] mM
[.1673] mM
[.08367] mM

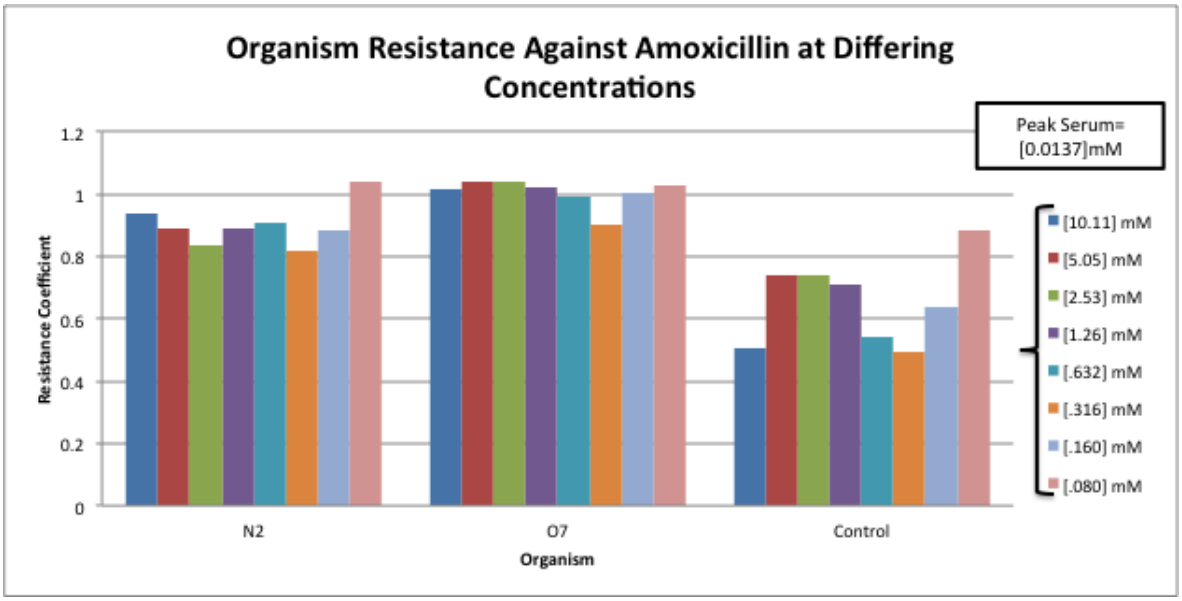


Amoxicillin

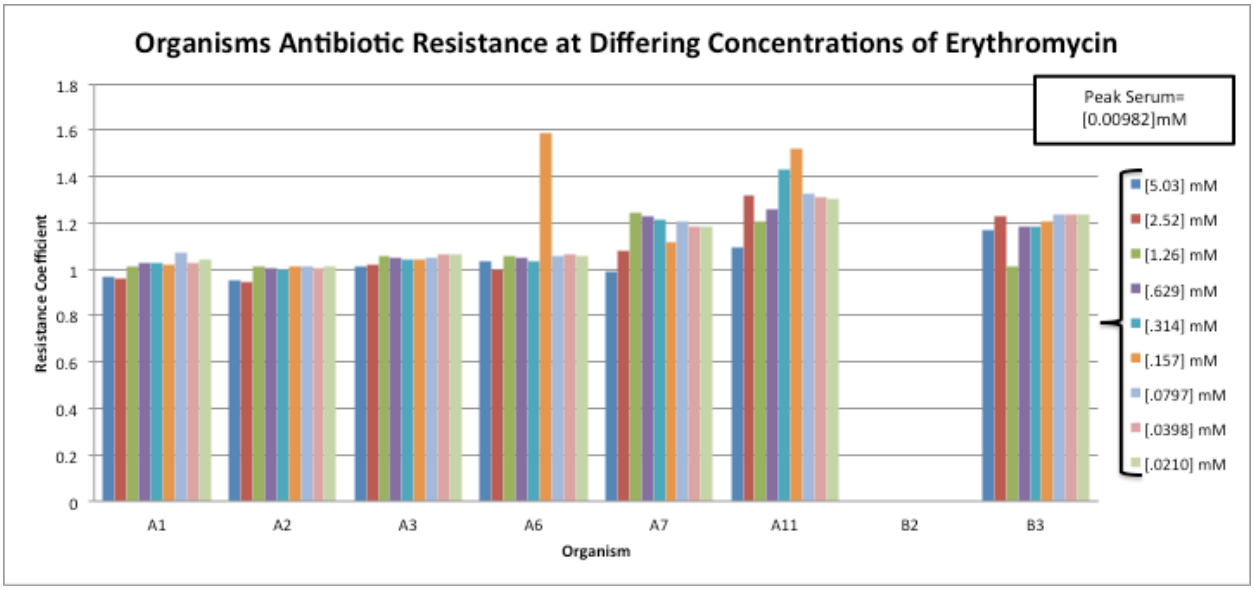


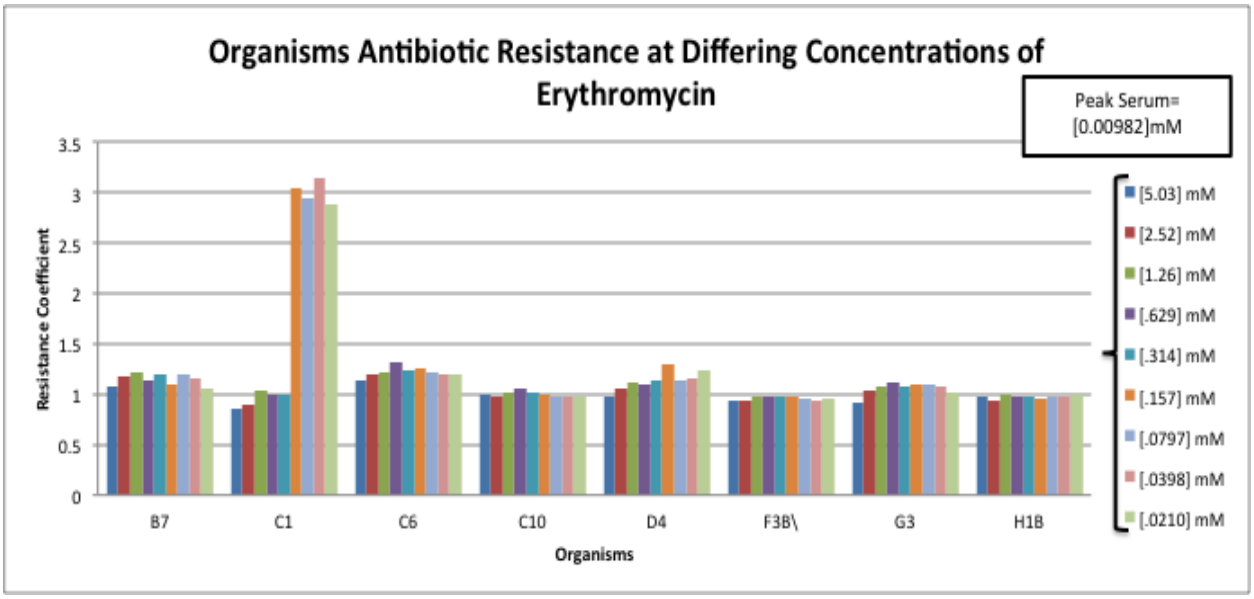

Organism Resistance Against Amoxicillin at Differing Concentrations
Peak Serum= [0.0137]mM
Resistance Coefficient
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
East Gemini B7
Eat Gemini C1
East Gemini C6
East Gemini C10
Lake Sag D4
Stumpf Lake F3B
West Gemini G3
West Gemini H1B
Organism
[10.11] mM
[5.05] mM
[2.53] mM
[1.26] mM
[.632] mM
[.316] mM
[.160] mM
[.080] mM

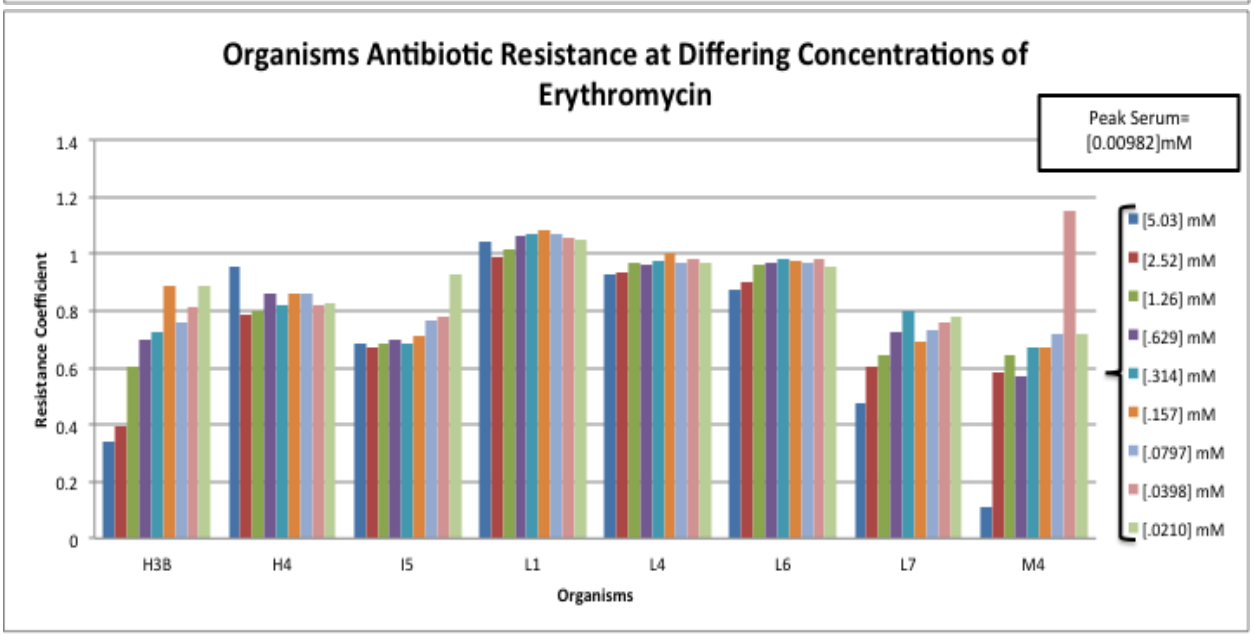

Organism Resistance Against Amoxicillin at Differing Concentrations
Peak Serum= [0.0137]mM
Resistance Coefficient
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
H3B
H4
I5
L1
L4
L6
L7
M4
Organism
[10.11] mM
[5.05] mM
[2.53] mM
[1.26] mM
[.632] mM
[.316] mM
[.160] mM
[.080] mM

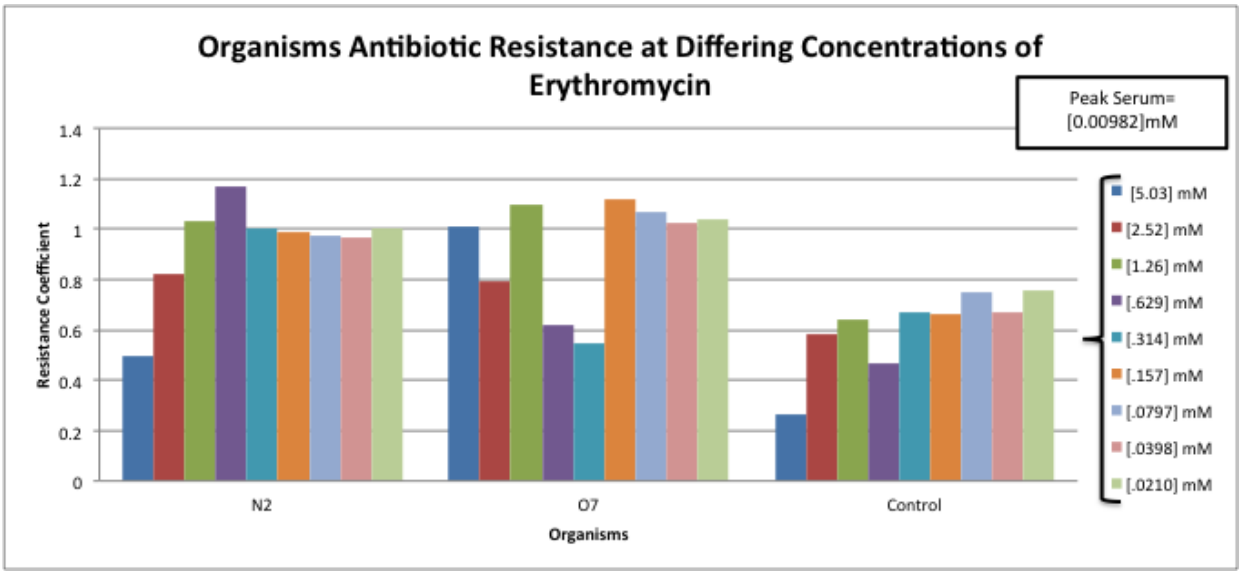


Erythromycin

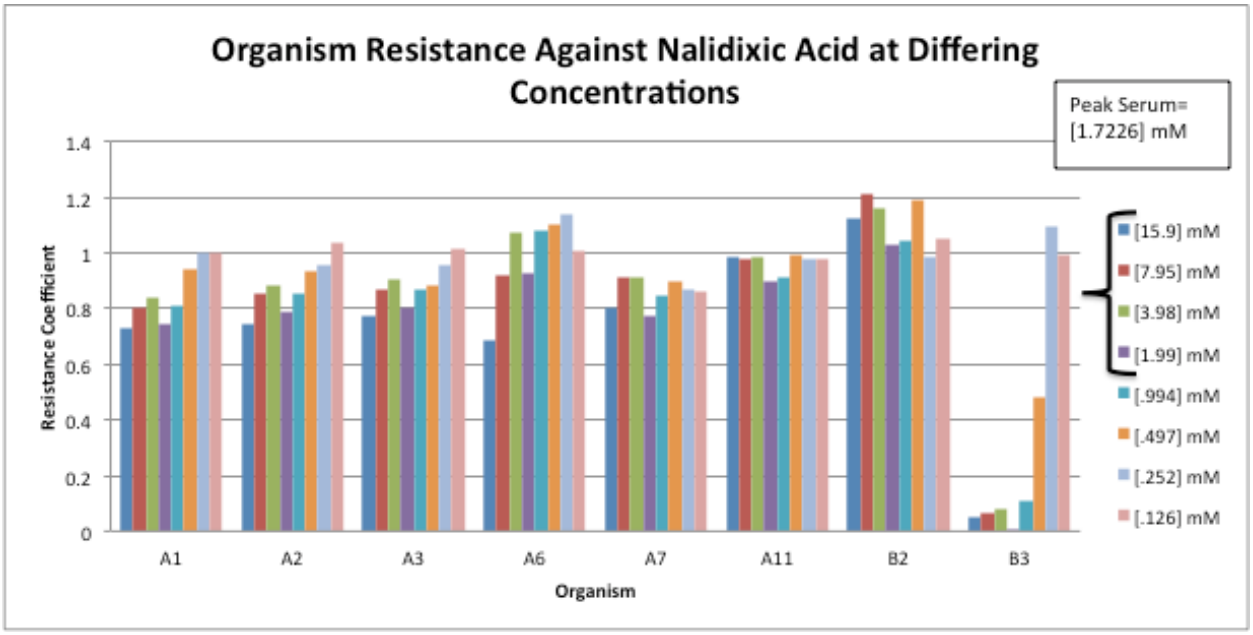


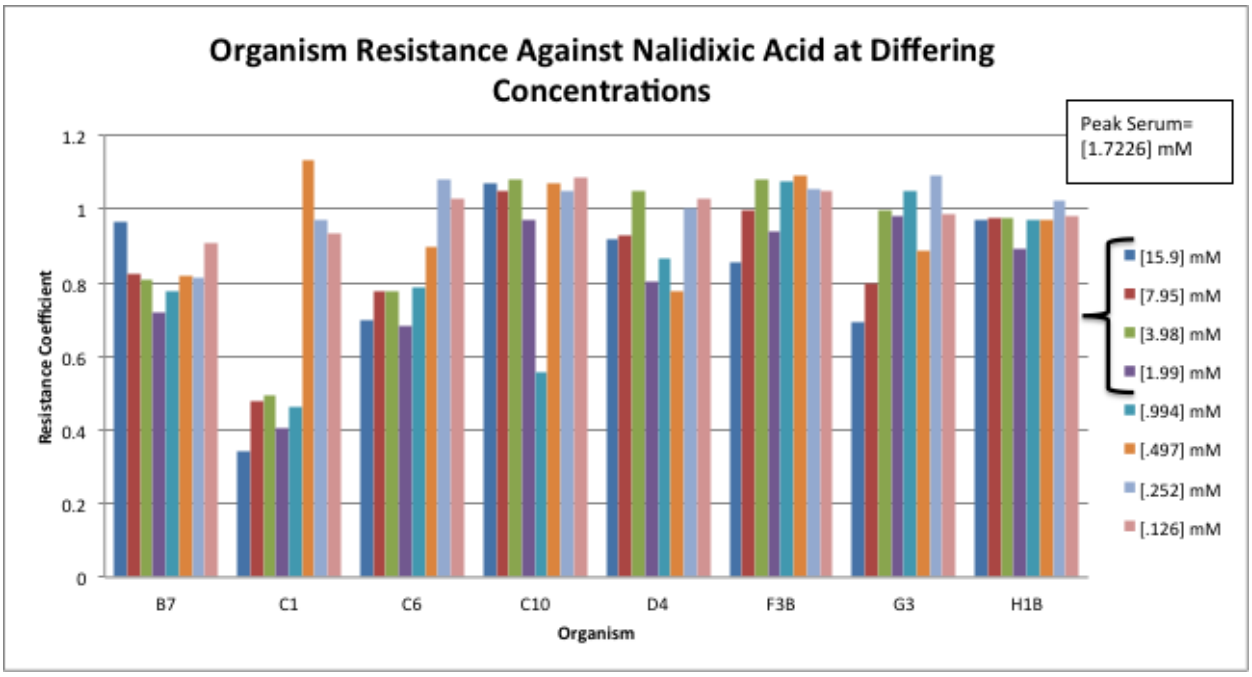

Organisms Antibiotic Resistance at Differing Concentrations of Erythromycin
Peak Serum= [0.00982]mM
Resistance Coefficient
3.5
3
2.5
2
1.5
1
0.5
0
B7
C1
C6
C10
D4
F3B\
G3
H1B
Organisms
[5.03] mM
[2.52] mM
[1.26] mM
[.629] mM
[.314] mM
[.157] mM
[.0797] mM
[.0398] mM
[.0210] mM

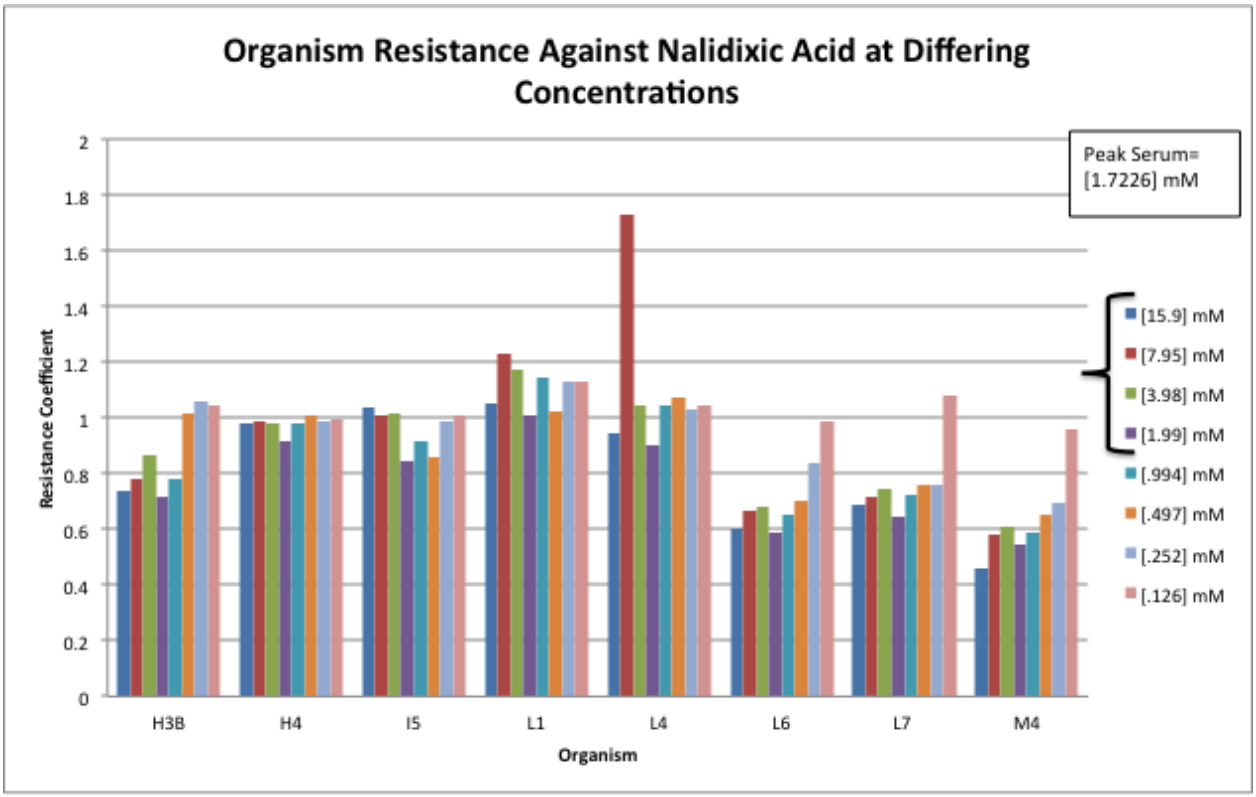

Organisms Antibiotic Resistance at Differing Concentrations of Erythromycin
Peak Serum= [0.00982]mM
Resistance Coefficient
1.4
1.2
1
0.8
0.6
0.4
0.2
0
H3B
H4
I5
L1
L4
L6
L7
M4
Organisms
[5.03] mM
[2.52] mM
[1.26] mM
[.629] mM
[.314] mM
[.157] mM
[.0797] mM
[.0398] mM
[.0210] mM

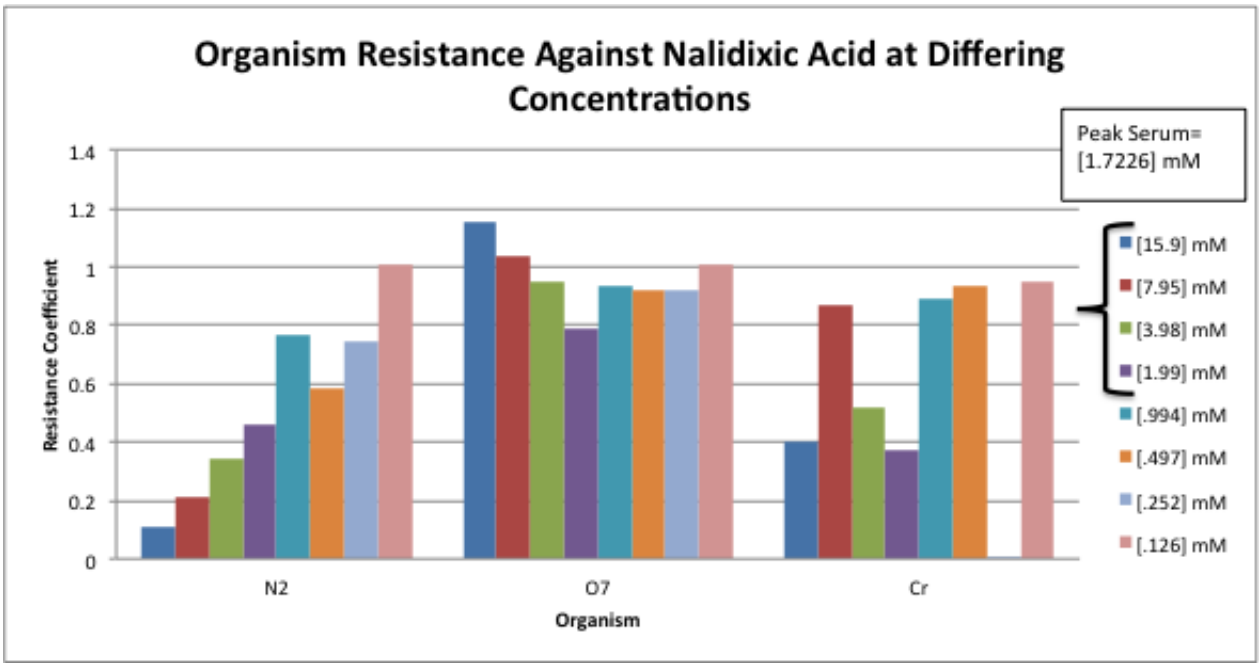


Nalidixic Acid

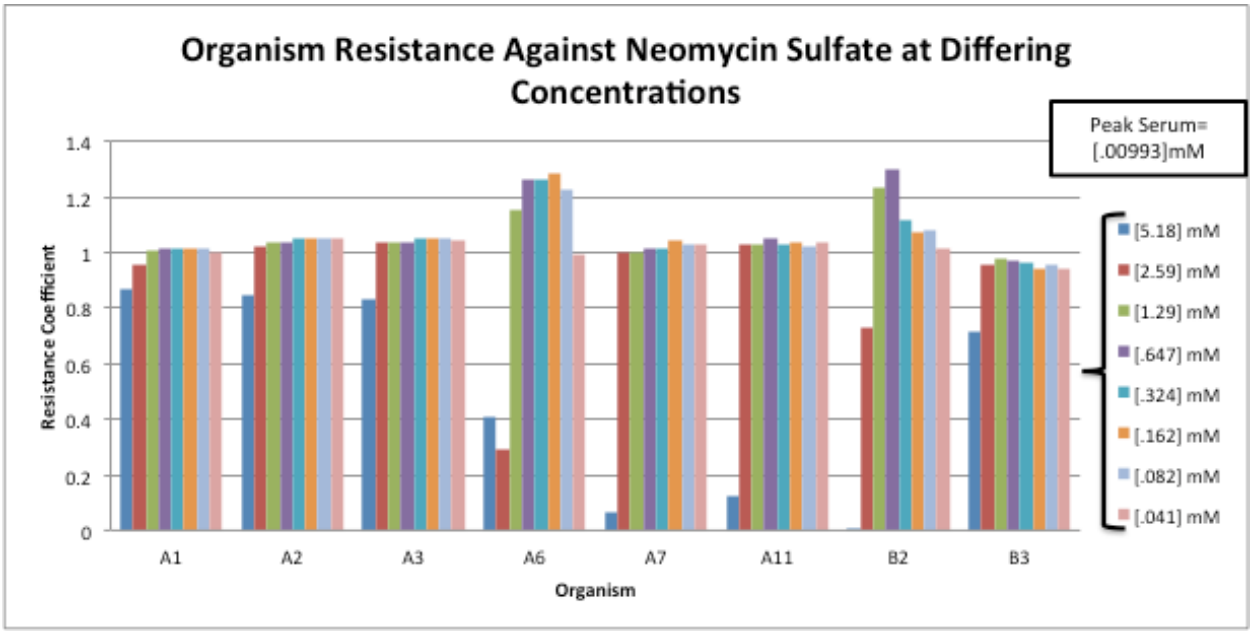


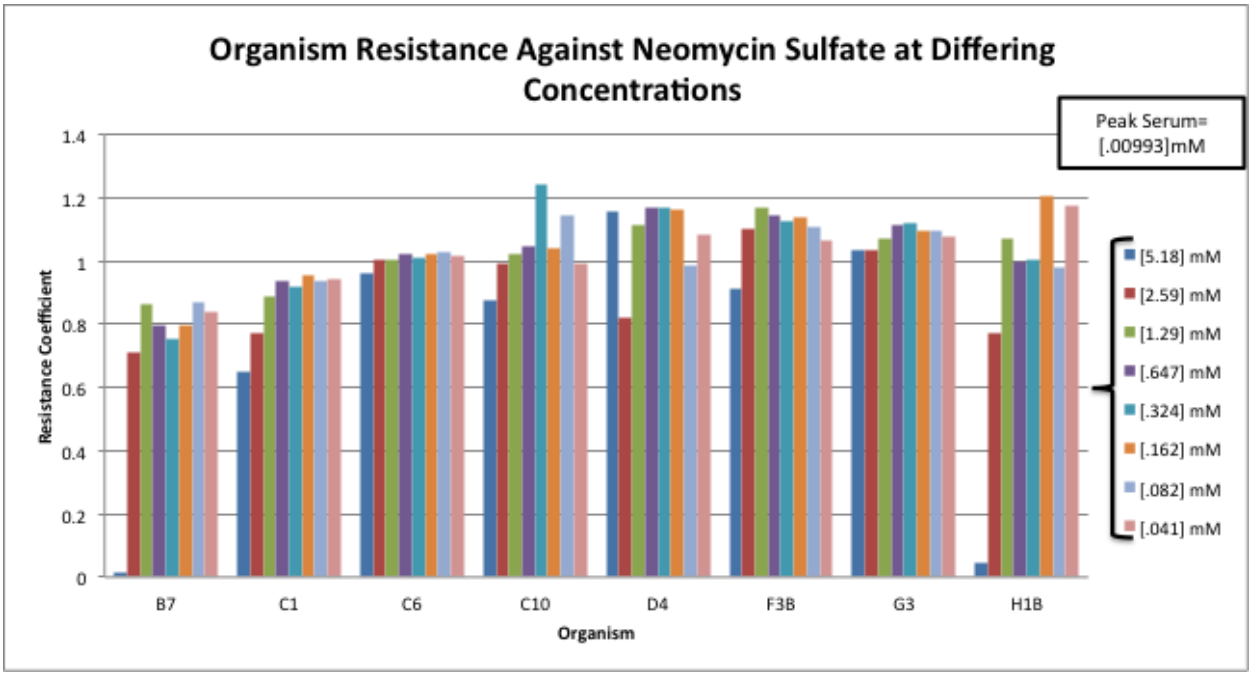

Organism Resistance Against Nalidixic Acid at Differing Concentrations
Peak Serum= [1.7226] mM
Resistance Coefficient
1.2
1
0.8
0.6
0.4
0.2
0
B7
C1
C6
C10
D4
F3B
G3
H1B
Organism
[15.9] mM
[7.95] mM
[3.98] mM
[1.99] mM
[.994] mM
[.497] mM
[.252] mM
[.126] mM

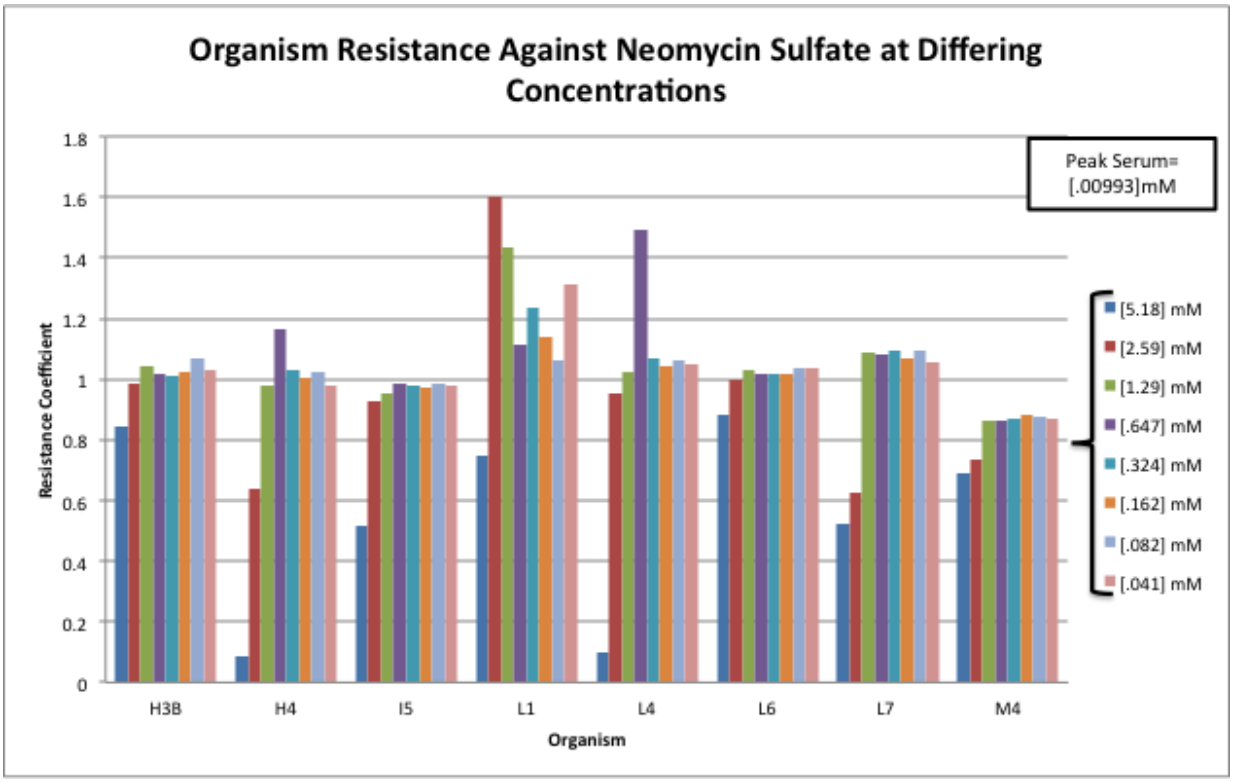

Organism Resistance Against Nalidixic Acid at Differing Concentrations
Peak Serum= [1.7226] mM
Resistance Coefficient
Organism
H3B
H4
I5
L1
L4
L6
L7
M4
[15.9] mM
[7.95] mM
[3.98] mM
[1.99] mM
[.994] mM
[.497] mM
[.252] mM
[.126] mM

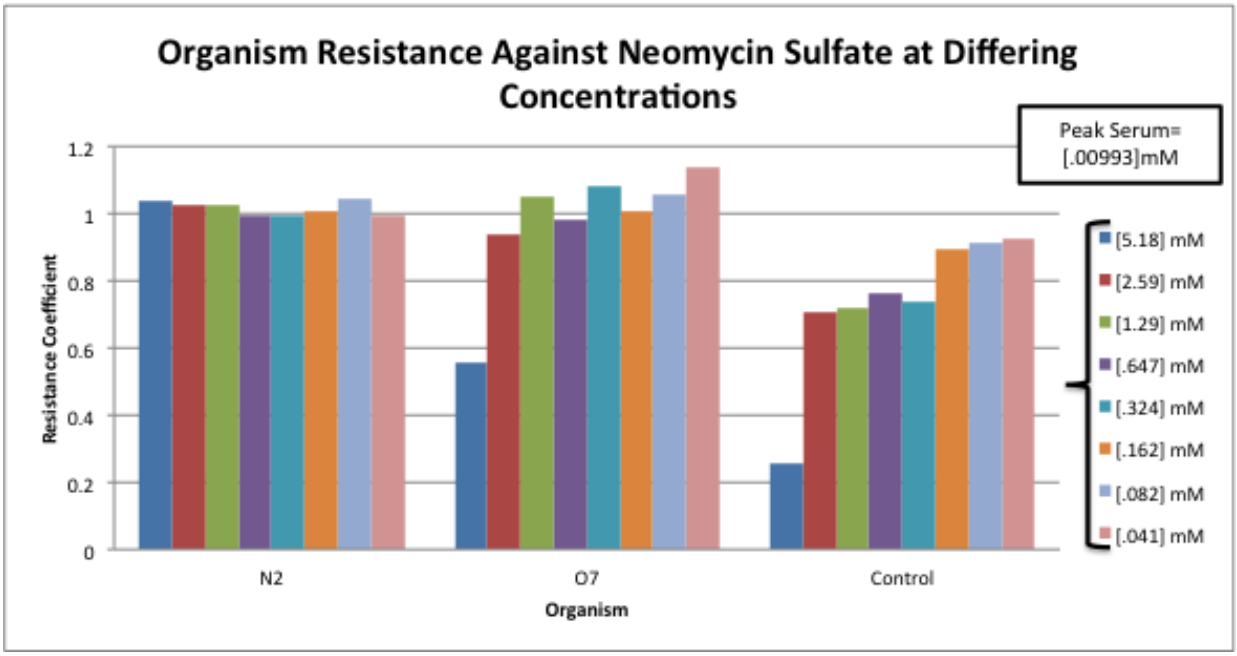

Organism Resistance Against Nalidixic Acid at Differing Concentrations
Peak Serum= [1.7226] mM
Resistance Coefficient
Organism
N2
O7
Cr
[15.9] mM
[7.95] mM
[3.98] mM
[1.99] mM
[.994] mM
[.497] mM
[.252] mM
[.126] mM

Neomycin Sulfate



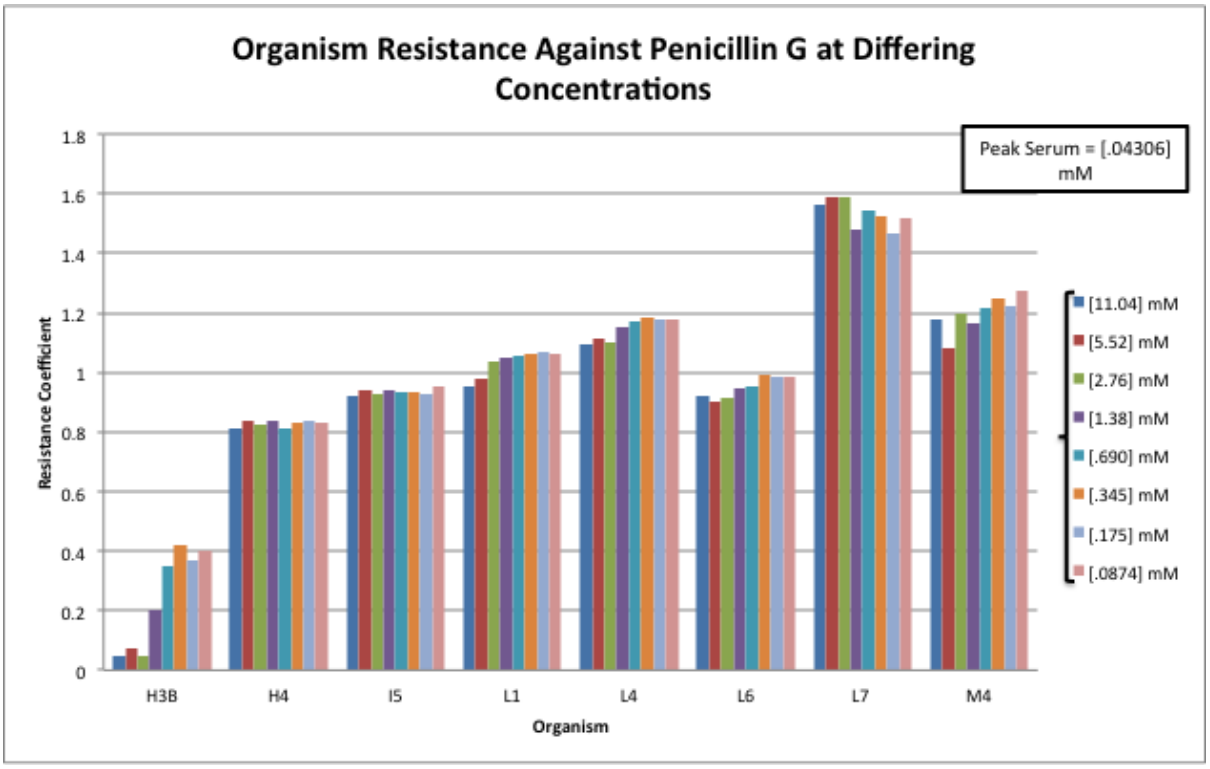


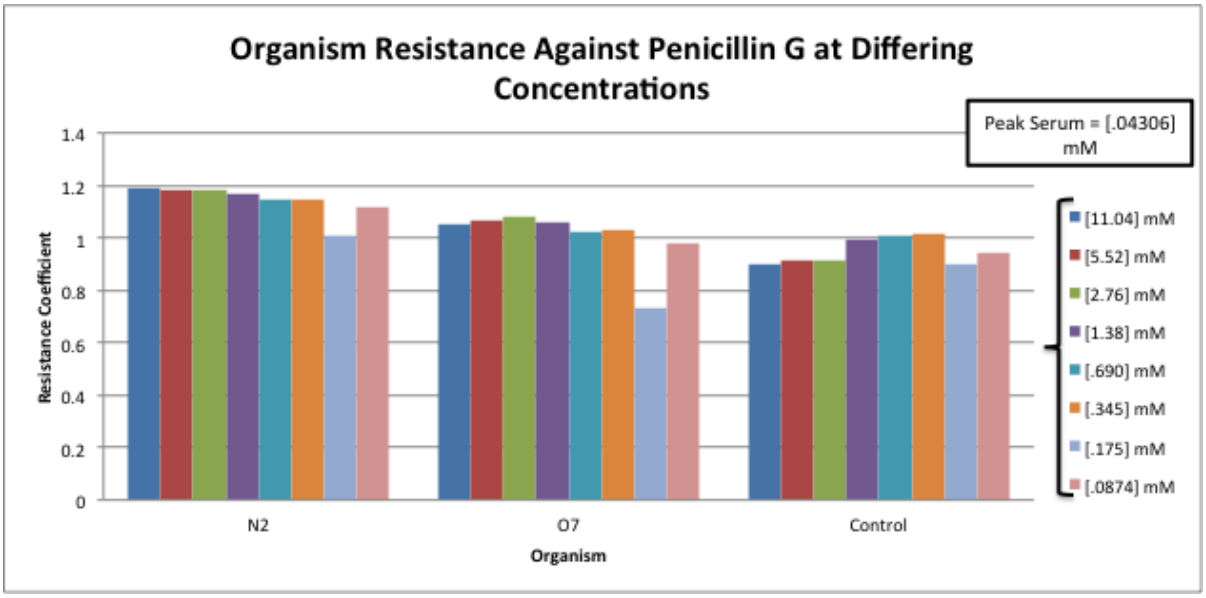

Organism Resistance Against Neomycin Sulfate at Differing Concentrations
Peak Serum= [.00993]mM
Resistance Coefficient
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
H3B
H4
I5
L1
L4
L6
L7
M4
Organism
[5.18] mM
[2.59] mM
[1.29] mM
[.647] mM
[.324] mM
[.162] mM
[.082] mM
[.041] mM


Organism Resistance Against Neomycin Sulfate at Differing Concentrations
Peak Serum= [.00993]mM
Resistance Coefficient
1.2
1
0.8
0.6
0.4
0.2
0
N2
O7
Control
Organism
[5.18] mM
[2.59] mM
[1.29] mM
[.647] mM
[.324] mM
[.162] mM
[.082] mM
[.041] mM

Penicillin G.

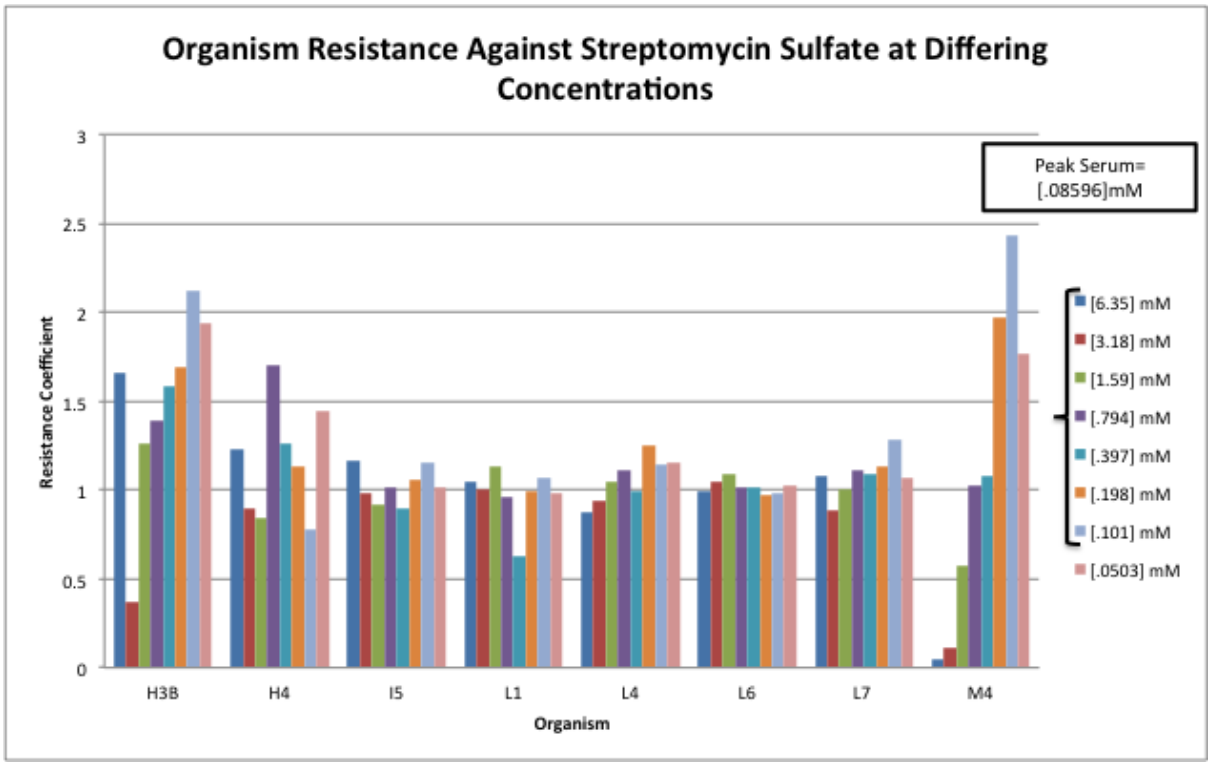
A1-B3, B7-H1B can be found on pages 13 and 14 respectively.

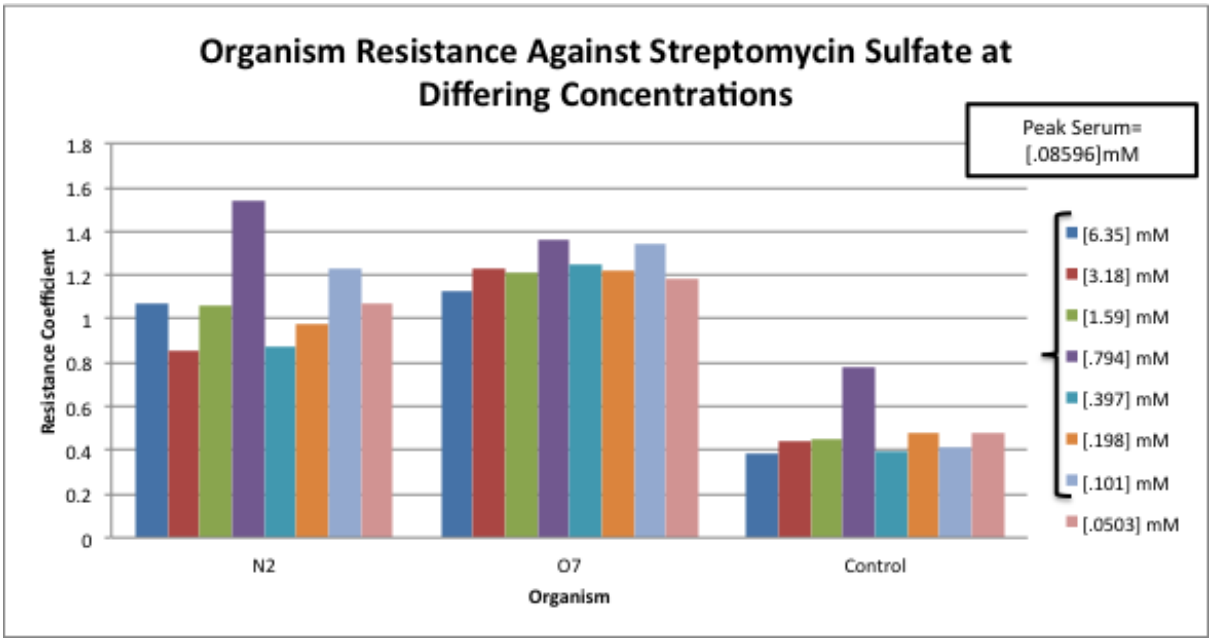




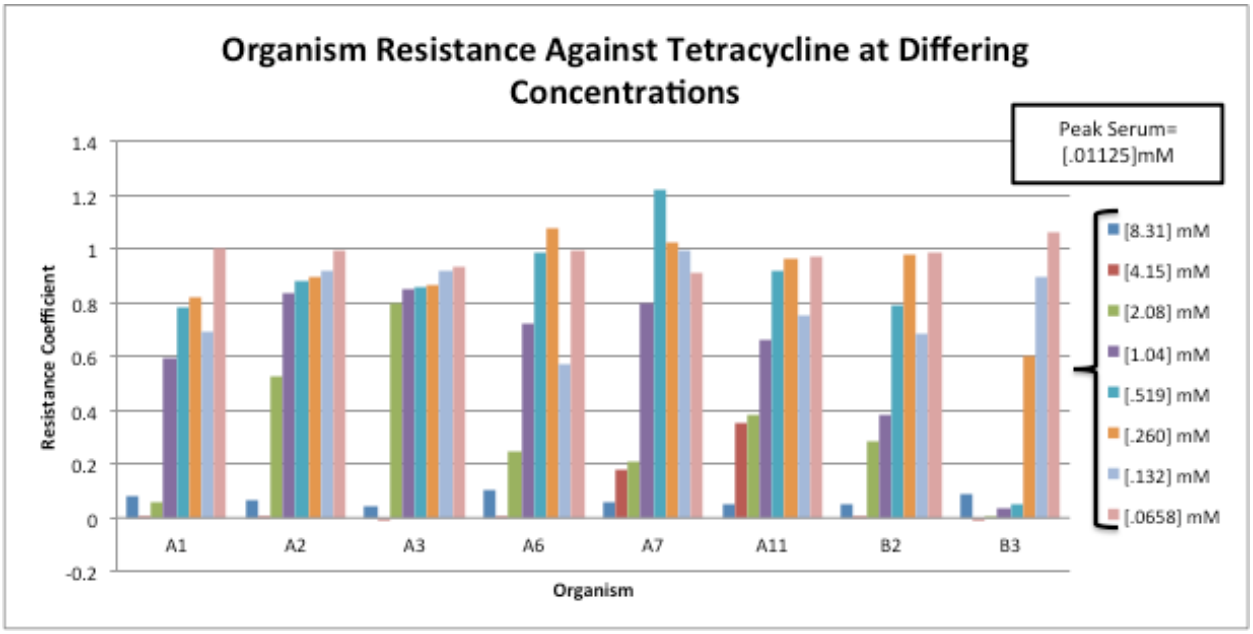
Streptomycin Sulfate

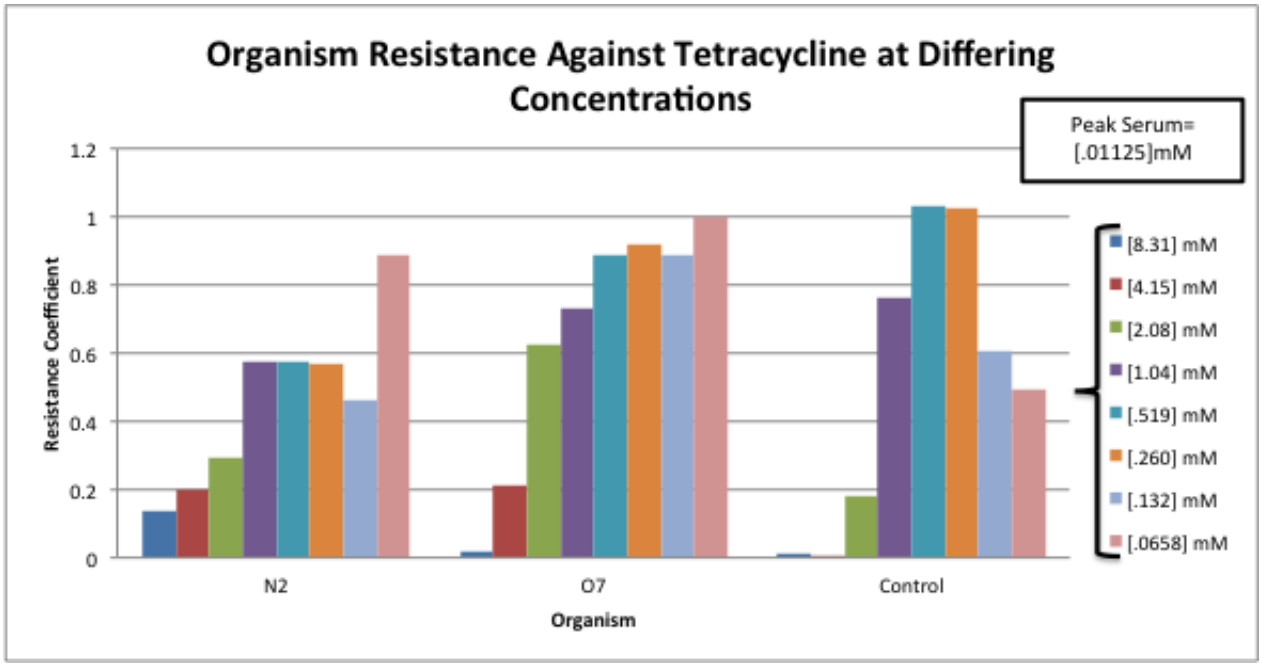
A1-B3, B7-H1B can be found on pages 11 and 12 respectively.

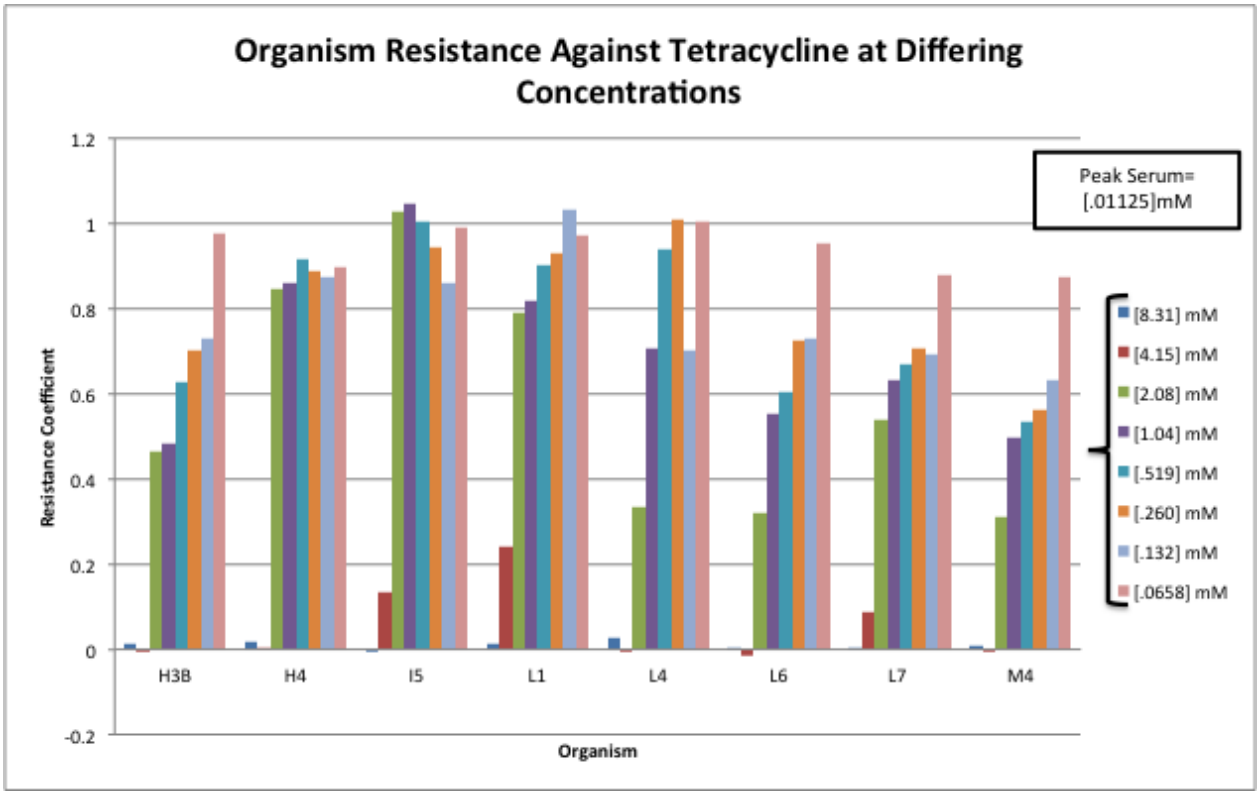


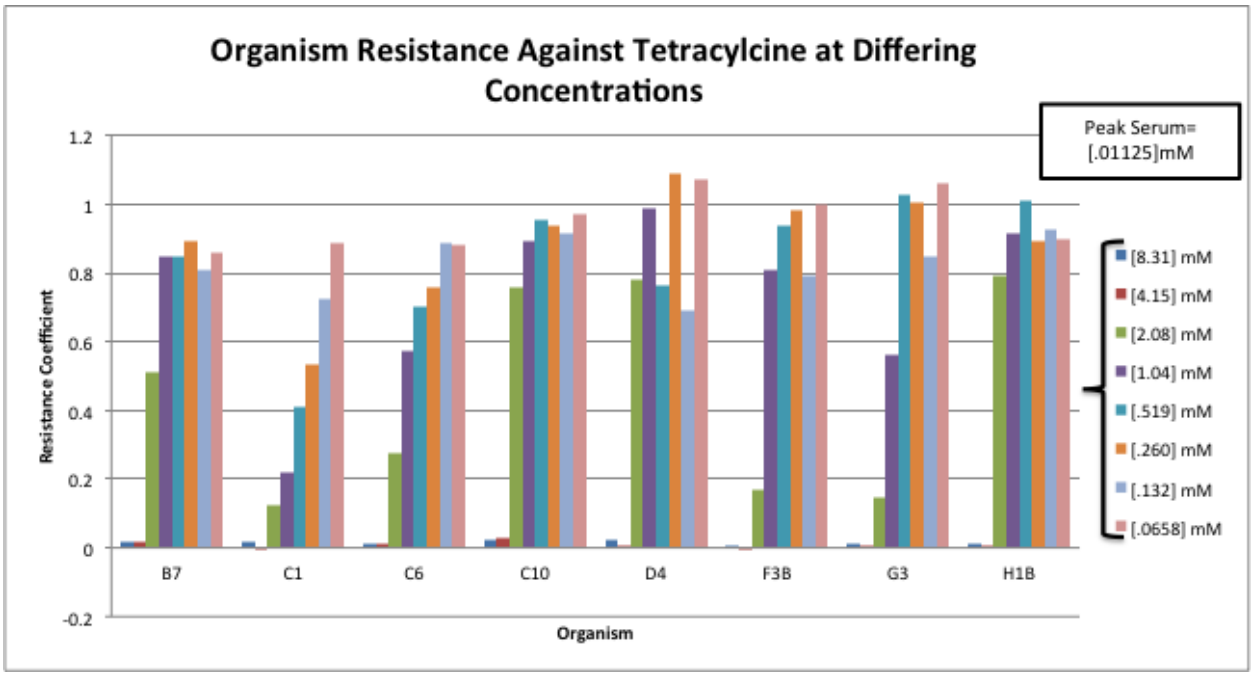


Tetracycline




Organism Resistance Against Tetracycline at Differing Concentrations
Peak Serum= [.01125]mM
Resistance Coefficient
1.2
1
0.8
0.6
0.4
0.2
0
N2
O7
Control
Organism
[8.31] mM
[4.15] mM
[2.08] mM
[1.04] mM
[.519] mM
[.260] mM
[.132] mM
[.0658] mM


Organism Resistance Against Tetracycline at Differing Concentrations
Peak Serum= [.01125]mM
Resistance Coefficient
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
H3B
H4
I5
L1
L4
L6
L7
M4
Organism
[8.31] mM
[4.15] mM
[2.08] mM
[1.04] mM
[.519] mM
[.260] mM
[.132] mM
[.0658] mM


Organism Resistance Against Tetracylcine at Differing Concentrations
Peak Serum= [.01125]mM
Resistance Coefficient
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
B7
C1
C6
C10
D4
F3B
G3
H1B
Organism
[8.31] mM
[4.15] mM
[2.08] mM
[1.04] mM
[.519] mM
[.260] mM
[.132] mM
[.0658] mM