A Summary of Three Antibiotic Resistant Bacteria Studies of Plaster Creek by Calvin College Biology 250 Students, Spring, 2014

YeaEun Lee
David Warners
Department of Biology, Calvin College

Introduction

Plaster Creek has come to be known as the most polluted stream in West Michigan, and in 2009 a watershed group, Plaster Creek Stewards, was formed at Calvin College to 'restore health and beauty to the Plaster Creek Watershed' (http://www.calvin.edu/admin/provost/pcw/). Biology 250 (Research Methods) is a semester-long class required of all Biology and Environmental Science students at Calvin College (http://www.calvin.edu/verge/allstories/bio-250-up-the-creek.html). In this sophomore-level class students learn how to conduct biological research by using the Plaster Creek Watershed as their laboratory. Research projects conducted by students in this class help inform the restoration efforts of Plaster Creek Stewards. Projects range from small scale laboratory focused topics (e.g. identifying environmental estrogens, assessing bacterial contamination, finding sources of excess nutrient loading, etc.) to broader field based studies (mapping rare plant species in the watershed, evaluating the effect of bridge overpasses on aquatic insects, etc.).

From Biology 250 studies, as well as other research conducted in previous years, we have learned that *E. coli* levels are extremely high in Plaster Creek. While these levels tend to peak with stormwater surges, they are typically found at levels higher than the state standards for full and partial human body contact (130 and 300 colony forming units per 100ml respectively – see http://www.michigan.gov/deq/0,4561,7-135-3313_3686_3730-11005--,00.html). Our sample with the highest recorded level of *E. coli* contamination had over 15,000 colony forming units per 100ml (recorded in summer, 2014). Identifying the sources of *E. coli* contamination is a major challenge for Plaster Creek Stewards. Another question elicited by these findings is, how much antibiotic resistance is present among the high levels of bacteria in Plaster Creek? In the spring of 2014, for the first time, three different groups of Biology 250 students addressed this question, each group with a slightly different approach. This paper summarizes their findings and offers recommendations for additional research to better understand antibiotic resistant bacteria in Plaster Creek.

Background

Antibiotics play a vital role in today's medical care system and have contributed to saving countless people from a variety of bacterial infections. With increasing emphasis on the importance of antibiotics throughout the world, their use has not been confined to the realm of human medicine but also has expanded to other areas, such as agriculture, industry, and wastewater treatment facilities. However, even though antibiotics have significantly contributed to the improvement of public health, the widespread use of antibiotics has raised a new dilemma: the emergence of various strains of bacteria that have developed antibiotic resistance. Thus, higher dosages of current antibiotics or development of completely new antibiotics is frequently required to treat infections caused by antibiotic resistant bacteria (ARB).

The emergence of ARB is not restricted to clinical settings but has spread more widely into the environment, and has become a particular concern in urban waterways. Research on a watershed in North-Central Indiana hypothesized that human activities could be a driving force in exacerbating this problem (Fincher 2009). For instance, urban water treatment plants are known to increase the release of antibiotics into the environment (Rizzo 2013). Also, runoff from antibiotic-treated animal manure often increases the level of antibiotic resistance in waterways immediately downstream from agricultural areas (Harnisz 2012). Such human activities are likely contributors to the presence of antibiotic resistant bacteria in streams – either as introduction points of ARB themselves, or as sources of high levels of antibiotics that can offer a strong selective force in a stream ecosystem that favors ARB (or both).

Overview of Projects

Studies conducted in Michigan have found the existence of ARB in several watersheds. For example, ARB were discovered in every water sample collected from river water in Oakland County, Michigan (Fogarty et al. 2005). Another study in the Red Cedar watershed (east of Lansing) found multiple *E. coli* strains resistant to a range of antibiotics. The presence of ARB in urban streams increases the likelihood for humans to contact resistant strains and subsequently contract antibiotic-resistant infections. These findings suggest a developing public health concern and highlight the importance of assessing ARB in the Plaster Creek Watershed, an area home to approximately 25% of the population of Kent County, draining most of southeast Grand Rapids and parts of Caledonia, Dutton, Ada and Gaines Townships.

Plaster Creek, which drains into the Grand River just south of downtown Grand Rapids, begins in the agricultural regions of Caledonia and Dutton, flows northward through suburban neighborhoods, commercial and industrial areas, and finally through lower income urban neighborhoods, including parks where there are many opportunities for human contact (FTC&H 2008, Plaster Creek Stewards unpublished data 2014). This is concerning because bacterial levels in the creek are very high – at most times considered unfit for both full and partial body contact (FTC&H 2008). Even though bacteria have been assessed and monitored in Plaster Creek for several years, no research has been conducted on the occurrence of ARB in Plaster Creek (Warners pers.com. 2014). Thus, investigating the presence of ARB in Plaster Creek and evaluating subsequent implications can offer motivation and guidance for restoring health to this local urban waterway

Three ARB studies were conducted in Plaster Creek during the spring of 2014 by three different groups of Biology 250 (Research Methods) students at Calvin College. The first group assessed the existence and distribution of antibiotic resistant *E. coli*, *Salmonella*, and *Enterococci* in two different locations along the creek; the second group investigated enteric bacterial concentrations and resistance at three different locations along the creek; and the last group studied potential agriculturally-related antibiotic resistant *E. coli* at several locations in Plaster Creek. In general, these studies all assessed the possible resistance of different types of bacteria to various antibiotics using the Kirby-Bauer disk diffusion method (see Figure 2). However, even though the purpose of each study was similar, each one evaluated a different hypothesis and each developed their own experimental design. In addition the groups focused on different bacteria and different antibiotics, and they employed different statistical analyses.

The purpose of this paper is to both summarize the three projects (see Appendix A for each summary) and highlight central similarities and contrasts among their results. We will also discuss the possible implications of this research to matters of public health in the Plaster Creek Watershed.

General Conclusions

Overall, all three ARB studies from Biology 250 reached a common conclusion: bacterial strains resistant to a variety of antibiotics do exist in Plaster Creek. The first study found that *Enterococci*, *Salmonella*, and *E. coli* were resistant to at least one of the four antibiotics tested, vancomycin, ampicillin, streptomycin, and cephalothin (Appendix A-I); the second study indicated complete resistance of *Enterobacteriaceae* to penicillin, as well as varying levels of resistance to triclosan and ceftriaxone (Appendix A-II); and the third study found multiple strains of *E.coli* that were resistant in varying degrees to tetracycline, ampicillin, erythromycin, and gentamicin (Appendix A- III).

The three studies hypothesized not only the existence of antibiotic resistant bacteria but also a connection between levels of ARB with various environmental factors. For example, the first and third studies tested an agricultural link to antibiotic resistance, predicting that higher levels of ARB would be found in the locations near dairy farms. The second study examined the effects of different weather conditions (dry and wet) on levels of ARB in the creek. However, the results from all three studies did not support the notion that either dairy farms or weather significantly influenced the relative abundance of ARB bacteria in Plaster Creek. While these possibilities were not completely discounted, an overall conclusion from these three projects is that antibiotic resistant bacteria are consistently present in the creek, across both space and time, and no clear connection to a particular location or land use activity was identified.

Other studies have also found varying levels of bacterial resistance to several antibiotics that were tested by the three Bio 250 studies. Specifically, high levels of resistance to ampicillin, tetracycline,

and streptomycin have been reported (Jones 2004; Brennan and Everman 2012). Also, these earlier reports examined the effect of wastewater treatment facilities and of agricultural runoff. Unlike results from Bio 250 studies on Plaster Creek, agricultural runoff was found to elevate the levels of ARB in stream water. In addition, these studies found higher levels of ARB associated with sewage treatment plants (Jones 2004; Brennan and Everman 2012). These effects of agricultural and sewage treatment runoff on ARB suggest that human use of antibiotics has become a major selective force, increasing levels of resistance in urban waterways. Even though there is no sewage treatment drainage into Plaster Creek, the selective agent for high levels of resistance could be leaking septic fields, animal waste from feedlots, or fields where manure is used as fertilizer. While it is important to realize that ARB are present in Plaster Creek, it is also important to note that ARB have been reported from many urban waterways and such reports appear to be on the increase throughout the US.

Yet, the findings of these three studies do raise concerns related to human health and the possibility of further development of ARB in Plaster Creek if remediation efforts are not undertaken. Furthermore, because of increased stormwater runoff and the ever-growing amount of antibiotics used in various medical, agricultural, and industrial settings, it seems likely that in the coming years Plaster Creek will experience elevated selective pressure for antibiotic resistance. Thus, it is critical to conduct additional research on antibiotic resistance in Plaster Creek, with a primary aim of identifying the main sources of the bacteria and the main sources of antibiotics that are acting as selective agents for this resistance. Addressing this issue will hopefully lead to a reduction in overall levels of bacteria in the stream, including levels of ARB. Furthermore, since the creek is readily accessible to the general public at many locations along its 14-mile stretch, growing antibiotic resistance would increase the possibility that Plaster Creek will become a significant health concern. Consequently, antibiotic resistance casts a shadow over public health in this watershed and should be approached with careful assessment and subsequent remediation efforts to better protect those who live along or interact with Plaster Creek.

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Appendices

Appendix A: Summaries of the Three Studies on Antibiotic Resistant Bacteria in Plaster Creek

I. The existence and distribution of antibiotic resistant *E. coli, Salmonella,* and *Enterococci* in Roosevelt and Paris parks

The first group hypothesized that antibiotic resistant bacteria would be found in Plaster Creek. They further predicted that the stream at Paris Park would show a higher level of ARB than that at Roosevelt Park, because of nearby dairy farms.

Methods

Sampling. Samples were collected intentionally within 24 hours of a rainfall event, in order to obtain higher bacterial counts. On April 3, 2014, a total of six water samples were collected at Paris Park and Roosevelt Park (Figure 1): three samples from the stream under the footbridge located just north of the 60th street entrance to Paris Park and another three samples from the stream adjacent to a large drainage pipe at Roosevelt Park (c. 100m north of Grandville avenue). Approximately 5 mL was collected from the surface of the stream for each sample. These samples were stored in a refrigerator overnight.

Culturing and identification. The samples were cultured in agar-based growth medium on April 4. Three selective agar bases were used to isolate the three different types of bacteria of focus: Coliscan Easygel for *E. coli*, Bile Esculin for *Enterococci*, and XLD for *Salmonella*. Three petri dishes for these three types of bacteria were generated from each sample, with a total of 18 petri dishes (Figure 1). The samples were transferred onto the agar using inoculating loops, and the plates were incubated at 36°C for 24 hours on April 5. After incubation, *E. coli* colonies cultured on Coliscan Easygel plates underwent secondary isolation, because many of the *E. coli* colonies identified were intermixed with other coliforms and difficult to isolate. For this secondary isolation, one largest *E. coli* colony from each plate was re-isolated on April 8. Six plates of Coliscan Easygel media were made, each with 4-5 mL of sterile water. Then one *E. coli* colony from each of the primary isolated plates was scooped out with an inoculating loop and spread over new Coliscan agar, using the quadrant streaking technique (Appendix A-1). The new *E. coli* plates were incubated overnight at 36°C for 24 hours.

Replicates. On April 8-9, after identification, a colony of each type of bacterium was picked up with an inoculating loop and mixed with liquid broth to make a broth culture. This was done for each type of bacteria from each water sample; the broth cultures generated in this way were incubated overnight at 36°C. From the broth cultures, 5 replicates for each type of bacterium from each water sample were made: a sterile cotton swab was dipped into a broth culture and dragged over the surface of a Mueller-Hinton agar (MHA) plate back and forth in a zig-zag formation. The MHA plates were incubated at 36°C for 24 hours on April 10.

Antibiotic testing. After incubation, Kirby-Bauer disks of four types of antibiotics (ampicillin, streptomycin, vancomycin, and cephalothin) were evenly placed on the surface of each MHA plate (Figure 2). The MHA plates with antibiotics were incubated at 36°C for 24 hours on April 12. In order to examine whether the bacterial species had shown resistance to any antibiotics used, the diameter of the circle of no bacterial growth around each disk, if any, was measured and compared to standards of each type of bacteria as defined by the Clinical and Laboratory Standards Institute (Appendix C-2).

Statistics. The results obtained were statistically analyzed by chi-square tests for each type of bacterium at each site, in order to compare the levels of antibiotic resistance between the two stream locations. R-commander was used to statistically assess the differences in *proportions* of the samples that showed antibiotic resistance between the two sites.

Results

The three selective agar bases successfully identified each of the three bacterial species: *E. coli* were identified as blue dots on Coliscan; *Enterococci* were identified as big black dots on Bile Esculin; and *Salmonella* were identified as gold-yellow dots or lines on XLD. Also, antibiotic resistance testing by Kirby-Bauer Disk Diffusion identified varying levels of resistance in the samples, as summarized for each bacterium below (Figure 3).

E. coli. After the primary isolation of *E. coli*, the samples from Paris Park produced one to four *E. coli* colonies per plate, and Roosevelt Park samples produced zero to three colonies per plate (Figure 4). The secondary isolation of *E. coli* successfully isolated pure *E. coli* colonies from all three samples collected at Paris Park. However, the secondary isolation of the sample from Roosevelt midstream failed to isolate *E. coli* so that the sample size of *E. coli* from Roosevelt Park was reduced to *two*. From each of the five secondary isolation plates, five replicates were created to test each antibiotic. An *average* of the disk diameter was taken of the five replicates to determine resistance. From Paris Park, one out of the three samples was resistant to streptomycin, and all three samples were resistant to vancomycin (naturally resistant; see Appendix C-2), ampicillin, and cephalothin. From Roosevelt Park, none of the samples were resistant to streptomycin and cephalothin, two out of two samples were resistant to vancomycin, and one out of two samples was resistant to ampicillin (Figure 7).

Enterococci. The three samples from Paris Park grew three to four colonies per plate, while samples from Roosevelt Park grew one to two per plate (Figure 5). At Paris Park, none of the samples showed resistance to streptomycin, all three samples were resistant to vancomycin and cephalothin, and two of the three samples were resistant to ampicillin (Figure 8)

Salmonella. *Salmonella* colonies were observed in samples from Roosevelt Park, but not in those from Paris Park. Those colonies isolated from Roosevelt Park were not distinctly separated: each of the three *Salmonella* plates showed one to three large groups of colonies (Figure 6). Of these three samples, none of them were resistant to streptomycin, and all three were resistant to vancomycin (naturally resistant; see Appendix C-2), ampicillin, and cephalothin (Figure 9).

Statistics. After determining antibiotic resistance, data comparing the two parks were analyzed with a Chi-square test using the *proportion* of samples that showed antibiotic resistance (Table 1). The data set for *Salmonella* was not analyzed with statistics because this type of bacteria was only found in Roosevelt Park. Almost all of the p-values obtained were greater than 0.05, which indicated no statistically significant differences in ARB levels between the two parks. The only p-value less than 0.05 was the resistance level of *E. coli* to cephalothin. This result indicates that antibiotic resistance of *E. coli* was significantly different between the two parks. However, the overall difference in ARB level between the two locations was not statistically significant.

Discussion

The results support the first hypothesis that antibiotic resistance exists in Plaster Creek. However, the p-values obtained through Chi-square analysis do not widely support the second hypothesis that the stream at Paris Park would show a higher level of ARB because of nearby dairy farms. However, the resistance of *E. coli* to cephalothin was significantly different between the two parks.

In terms of the distribution of bacteria, *Salmonella* were identified at Roosevelt Park but not at Paris Park. The existence of *Salmonella* found only at Roosevelt Park suggests the source lies somewhere between these two sampling locations. *Salmonella* has been reported to be carried and transmitted by the fecal matter of most domestic animals (ducks, cattle, dogs, cats, and chickens) (Vermont Department of Health 2014). Therefore if identification of the source of these bacteria is desired, possible entry points involving such animals could be explored between Paris and Roosevelt Parks.

Antibiotic resistance testing showed that all three types of bacteria from both parks were almost completely susceptible to streptomycin but completely resistant to vancomycin. *Salmonella* and *E. coli* were expected to have resistance to vancomycin because of their inherent resistance to the antibiotic (Appendix C-2). One possible source of the resistance of *Enterococci* to vancomycin could be from hospitals where vancomycin is extensively used to treat serious microbial infections today. For *Enterococci* and *E. coli*, we found some variation in resistance to ampicillin and cephalothin between the samples from each park. However, this difference in the level of antibiotic resistance between the two parks was not statistically significant (p-values >0.05). The only significant difference we found between the two parks was resistance of *E. coli* to cephalothin (Figure 7). *E. coli* from all three samples at Paris Park were resistant to cephalothin, whereas none of the *E. coli* samples from Roosevelt Park were resistant to this antibiotic. This result is unexpected because the creek flows from Paris Park to Roosevelt Park, and we would expect resistance found in the upper reaches to persist and also be present

in the downstream areas. One possible explanation for this difference could be that as more water volume is added to the stream, the resistant bacteria become more diffuse and are less likely to show up in downstream water samples. Further research is needed to better understand this finding.

Limitations. For this study, only three samples were collected from each park, and five replicates were made from each sample. However, those replicates were not independent because only *one* bacterial colony was used to generate each sample, rather than utilizing one colony for each replicate. Because a Chi-square test was run by using the "proportions" of the samples that showed antibiotic resistance, and because the replicates were not independent, only a sample size of three was utilized, which limited the power of our statistical comparison. In addition, low bacterial levels in the water samples limited the statistical power. Only two to three bacterial colonies were identified from each agar plate. To obtain higher bacterial levels in water samples, they need to be collected shortly after a heavy rainfall. Higher bacterial concentrations in the samples will help generate the needed number of independent replicates and thus increase the statistical power of the experimental results.

II. Investigation of Enteric Bacterial Concentration and Resistance in Plaster Creek

The second group hypothesized that antibiotic resistant bacteria would be present in Plaster Creek. They further predicted that greater quantities of Enterobacteriaceae would be found in the lower reaches of the stream and that bacterial levels would be higher following a heavy rainfall.

Methods

Sampling. The samples were collected at three different reaches of Plaster Creek: Shadyside Park, Ken-O-Sha Park, and Roosevelt Park. At each reach, three 45 mL water samples were collected in both a dry period (more than 48 hours after the last rainstorm) and a wet period (within 24 hours of a rainstorm with accumulation of at least 0.5 in of precipitation). A total of 18 samples were collected.

Culturing and identification. The samples were subjected to serial dilutions and then cultured on EMB agar plates for the growth of Enterobacteriaceae. After a 48-hour incubation, the total number of bacterial colonies was counted in order to determine the variance of bacterial levels at different locations in Plaster Creek. The procedure was repeated for both the dry and wet samples.

Antibiotic testing. Antibiotic resistance was tested on the samples from Ken-O-Sha Park by the Kirby-Bauer disk diffusion method. Five distinct colony-forming units (cfu's) from each of the wet and dry Ken-O-Sha samples were isolated and plated, giving a total of ten plates. Kirby-Bauer disks of five different antibiotics were applied to each of the ten plates: ceftriaxone, doxycycline, triclosan, penicillin, and imipenem. After a 48-hour incubation, the zone of inhibition around each antibiotic disk was analyzed.

Statistics. An ANOVA test was conducted to determine whether there were statistically significant differences in bacterial levels between the reaches and between the wet and dry samples. Individual t-tests were run for each location to compare bacterial abundance between weather conditions. A Chi-square analysis was run to examine whether the ten plated bacteria from Ken-O-Sha Park had significant resistance to each antibiotic tested In this project simulated resampling was conducted because of the relatively small sample size.

Results

ANOVA analysis. Comparing *Enterobacteriaceae* counts between all three locations and between weather conditions, the ANOVA test gave statistical significance at a significance level of $\alpha = 0.05$ (Figure 10). A significant p-value of 0.000012 was found when testing for differences in bacterial levels between locations, with Ken-O-Sha Park having the highest bacterial levels. Also, the differences in bacterial levels between the dry and wet samples were statistically significant ($p < 0.0000001$), with greater amounts of bacteria in the wet samples than the dry samples at all locations.

Individual t-tests. At each location, wet samples showed a greater amount of *Enterobacteriaceae* than dry samples did. The p-values for the three sites were significant at $\alpha = 0.05$ significant level (table 2).

Chi-square analysis. *Enterobacteriaceae* in both the wet and dry samples from Ken-O-Sha Park had antibiotic resistance developed, supported by a significant p-value of 0.0005. Among the five types of antibiotics tested, the greatest resistance was found in penicillin. Only two colonies displayed

resistant to triclosan, and one colony displayed intermediate resistance to ceftriaxone (Figure 11). Also, another Chi-square test that compared the level of observed resistance between wet and dry samples gave an insignificant p-value of 0.4809.

Discussion

The results did not clearly support the hypotheses that greater quantities of *Enterobacteriaceae* would be found in the lower reaches of the stream, near Roosevelt Park. The bacterial levels at Ken-O-Sha (mid-stream location) were consistently higher than those at Shadyside Park and Roosevelt Park. However, the increased total bacterial levels in the wet samples compared to the dry samples at all locations supported the hypothesis that bacterial levels would be higher following a rainfall. This observation could be explained by increased bacterial runoff from residential lawns, tile drains, or the soil near the stream banks following rainfall events.

Antibiotic resistant *Enterobacteriaceae* found at significant levels in both wet and dry samples supported the third hypothesis that antibiotic resistant bacterial strains are present in Plaster Creek. In both wet and dry conditions, *Enterobacteriaceae* were most resistant to penicillin. One possible source of these resistant strains could be dairy farms, particularly if they regularly administer antibiotics such as penicillin (Food and Drug Administration 2006). Also, if antibiotic-rich manure is spread onto farm fields and if some of it drains from these fields into the nearest waterway, penicillin or penicillin-resistant bacteria could eventually become carried into the main channel of Plaster Creek. For those enteric bacteria that showed resistance to triclosan, their resistance could be attributed to the high amount of triclosan antibiotics used in various hygienic goods like hand soaps (Drury et al. 2013). Just as the introduction of penicillin-resistance, triclosan itself or bacterial strains resistant to it could have been entering Plaster Creek through the sewage system. Overall, this research revealed the presence of antibiotic resistance to penicillin and triclosan, and future studies could be done to determine the sources of antibiotics or antibiotic resistant bacterial strains in the creek.

III. A study of potential agriculturally-related antibiotic resistant *E. coli* in several areas of Plaster Creek

The third group hypothesized that a presence of E.coli resistant to common antibiotics would be observed in Plaster Creek. They also predicted that this resistance would be related to the presence of dairy and agriculture farms in the watershed.

Methods

Sampling. Samples were obtained at three different locations along the creek: Shadyside Park, Paris Park, and under the Madison Avenue bridge. At each location, three 5 mL samples and a control were collected from the close, middle, and far banks, placing each sample into an Easygel® media bottle. Upon returning to the lab, the gel medium in each bottle was poured into a fresh, sterile petri dish, and the plates were incubated for 48 hours.

Culturing and identification. Three colonies were randomly selected from each plate to be cultured. For those plates that had fewer than three colonies, as many colonies as were available were isolated. Streak plates out of each individual selected colony were made on Mueller-Hinton plates. After a 48-hour incubation, a single colony of the bacteria was placed into a tube of Tryptic Soy Broth (TSB). Two plates were made from each tube by plating 100 μL on each Mueller-Hinton plate.

Antibiotic testing. Four different types of antibiotics that belong to different classes of drugs were tested: tetracycline, ampicillin, erythromycin, and gentamicin. These antibiotics all have human uses and applications, specifically with FDA approval for use in food-producing for livestock. Each Mueller-Hinton plate received two of the four antibiotic disks in order to prevent crowding and were incubated for 48 hours. After the incubation was complete each plate was measured for zones of inhibition around each disk to determine resistance. The industry standard guidelines for zone of inhibition measurements were used to determine susceptibility, intermediate resistance and resistance for each antibiotic are provided on the graphs in Figures 12-15.

Statistics. A Chi-square analysis was done to determine if there was a significant difference in the amount of resistance between locations. Graphs were generated to visually represent the resistance, sorted by both antibiotic type and location.

Results

Shadyside Park. E. coli strains from all three samples showed complete resistance to Ampicillin and Erythromycin but complete susceptibility to tetracycline and gentamicin (Figure 16).

Paris Park. Only two of the three samples contained *E. coli* colonies resistant to ampicillin and erythromycin. *E. coli* in the sample from the middle bank showed resistance also to tetracycline. By contrast, all three samples showed complete susceptibility to gentamicin (Figure 17).

Madison Avenue Bridge. Each of the three samples showed varying levels of resistance to all four antibiotics tested (Figure 18). Two of the three *E. coli* colonies from the close bank, two of the three from the middle bank, and only one of the three from the far bank were resistant to ampicillin. Only one of the three *E. coli* colonies from the close bank showed resistance to gentamicin, whereas the rest of the colonies from the close, middle, and far banks were susceptible to the antibiotic. All three *E. coli* colonies from the close bank, two of the three from the middle bank, and two of the three from the far bank showed resistance to erythromycin. All *E. coli* colonies, except for the one from the close bank, were susceptible to tetracycline.

Discussion

The results confirmed that there is an abundant amount of antibiotic resistant strains of *E. coli* in Plaster Creek. Of even more biological importance is the finding of multiple strains of *E. coli* that are resistant to multiple antibiotics identified at every location. However, the existence of resistant *E. coli* did not appear to be linked to the presence of dairy farms. Thus, the results did not strongly support the prediction of an agricultural link to antibiotic resistance.

In summary, high levels of antibiotic resistant *E. coli* were isolated from Plaster Creek. Bacteria were mainly resistant to ampicillin and erythromycin, but also generally susceptible to tetracycline and gentamicin. This resistance was found equally across all three locations, making a link between the various agricultural establishments along the creek to the amount of resistance unclear. In conclusion, several multi-resistant strains of *E. coli* at each location lead to greater concern for the health of the stream and the implications this may have on the surrounding communities and neighborhoods.

Appendix B: Figures and Tables

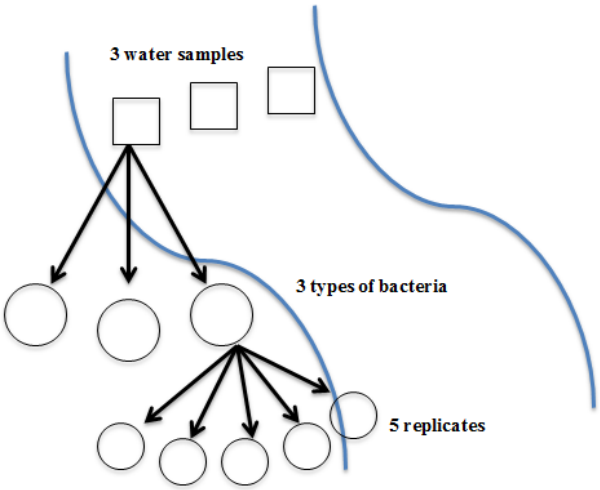

Figure 1. General scheme of our experiment. three water samples were obtained from each park, three types of bacteria were isolated from each sample, and five replicates for each bacterial type were made.

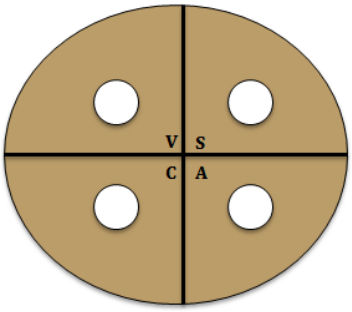

Figure 2. General scheme of Kirby-Bauer disk diffusion test; vancomycin, streptomycin, cephalothin, and ampicillin were evenly placed on each plate.

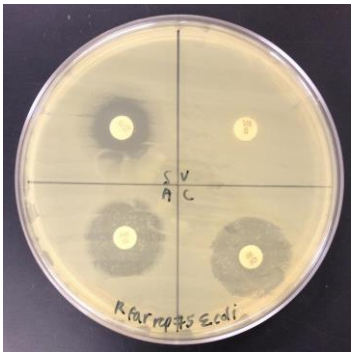

Figure 3. An example of the results of the Kirby-Bauer Disk Diffusion Test for a sample of E. coli from Roosevelt Park

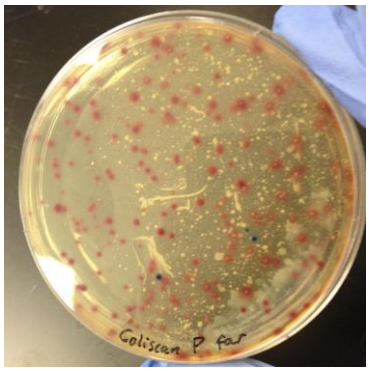

Figure 4. An example of isolated *E. coli* colonies in Coliscan EasyGel from a sample from Paris Park

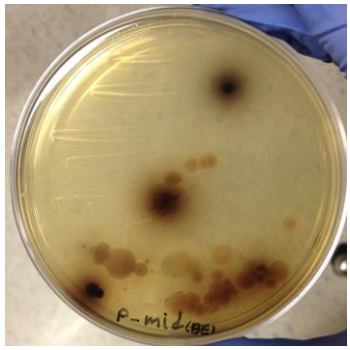

Figure 5. An example of *Enterococci* colonies identified by Bile Esculin from a sample from Paris Park

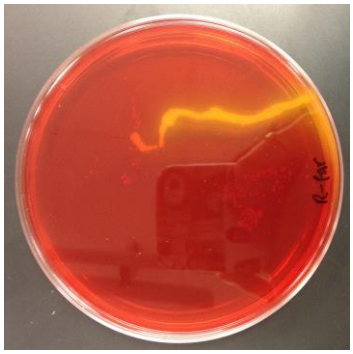

Figure 6. An example of *Salmonella* colonies isolated with XLD agar from a sample from Roosevelt Park

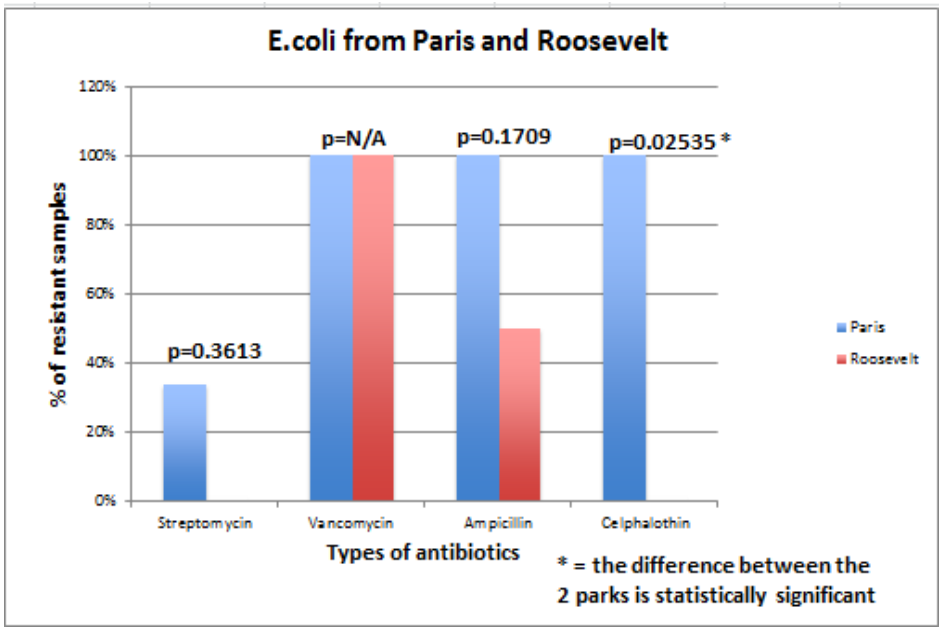

Figure 7. The proportion of *E. coli* samples that showed resistance to each type of antibiotics. P-values from a Chi-square test are indicated above the bars.

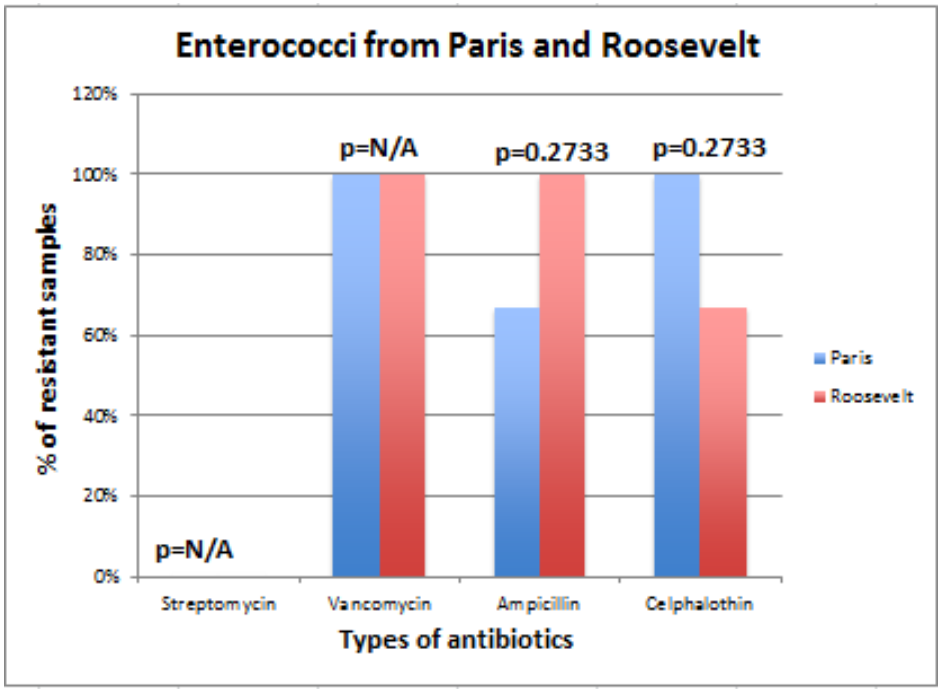

Figure 8. The proportion of *Enterococci* samples that showed resistance to each type of antibiotics. P-values from a Chi-square test are indicated above the bars.

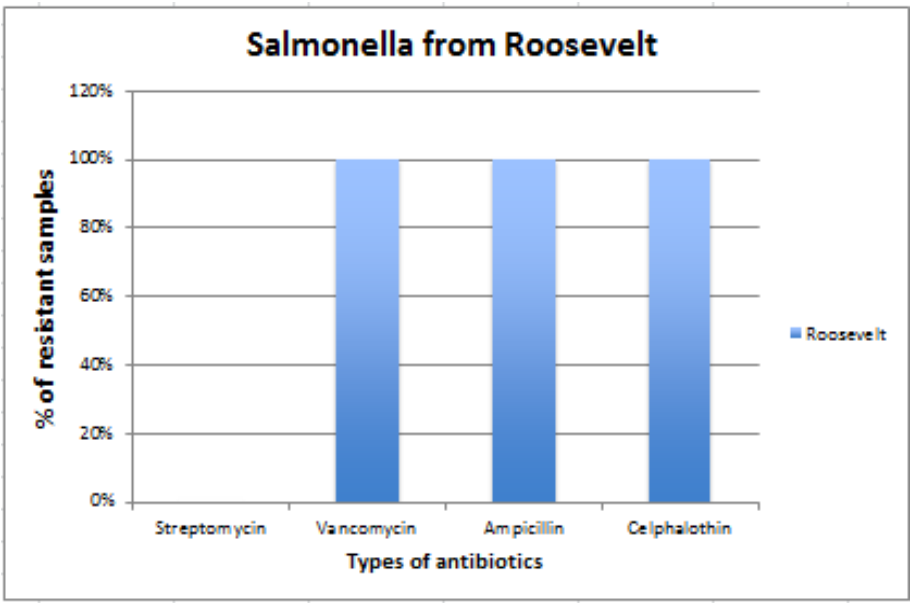

Figure 9. The proportion of *Salmonella* samples that showed resistance to each type of antibiotics. P-values from a Chi-square test are not indicated because *Salmonella* were only found in the samples from Roosevelt Park.

```
Analysis of Variance Table

## Response: log10(cfu)
##                  Df Sum Sq Mean Sq F value  Pr(>F)
## location          2   0.86    0.43    33.7 1.2e-05 ***
## weather           1   8.68    8.68   683.9 6.0e-12 ***
## location:weather  2   0.19    0.10     7.5  0.0077 **
## Residuals        12   0.15    0.01
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Figure 10. ANOVA of bacterial counts based on location and weather

```
            Ceftriaxone Doxycycline Imipenem Penicillin Triclosan
Resistant             1           0        0          8         2
Susceptible           8           9       10          1         8

 Pearson's Chi-squared test with simulated p-value (based on 2000
 replicates)

data:  ResistanceTable
X-squared = 28.16, df = NA, p-value = 0.0004998
```

Figure 11. Chi-square analysis of resistance based on antibiotic

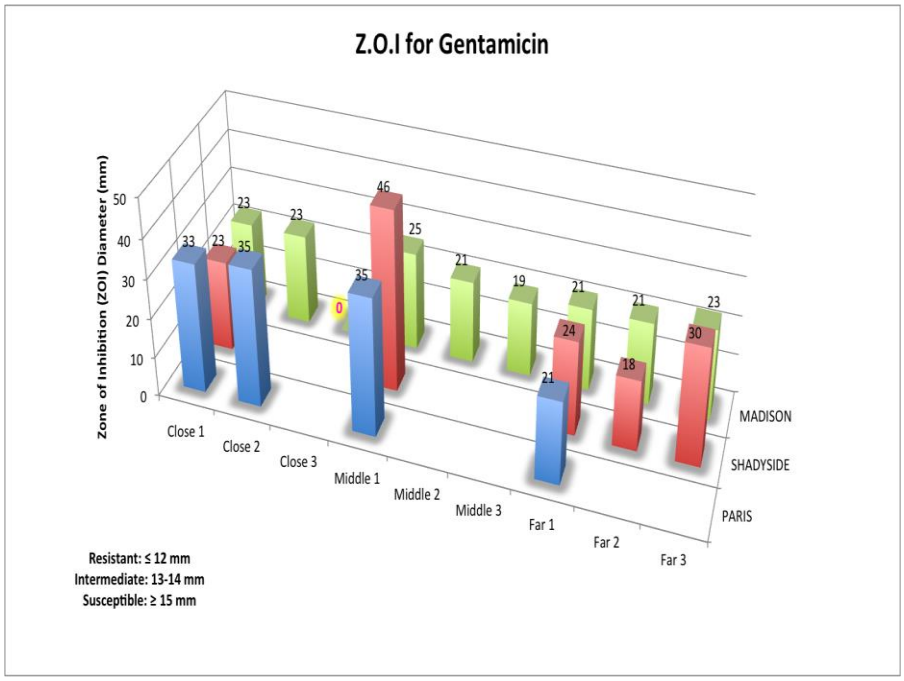

Figure 12. The above, left chart shows the diameter of the Zone of Inhibition (Z.O.I.) in millimeters for each of the colonies tested with Gentamicin. Each number highlighted in yellow represents a colony that is resistant.

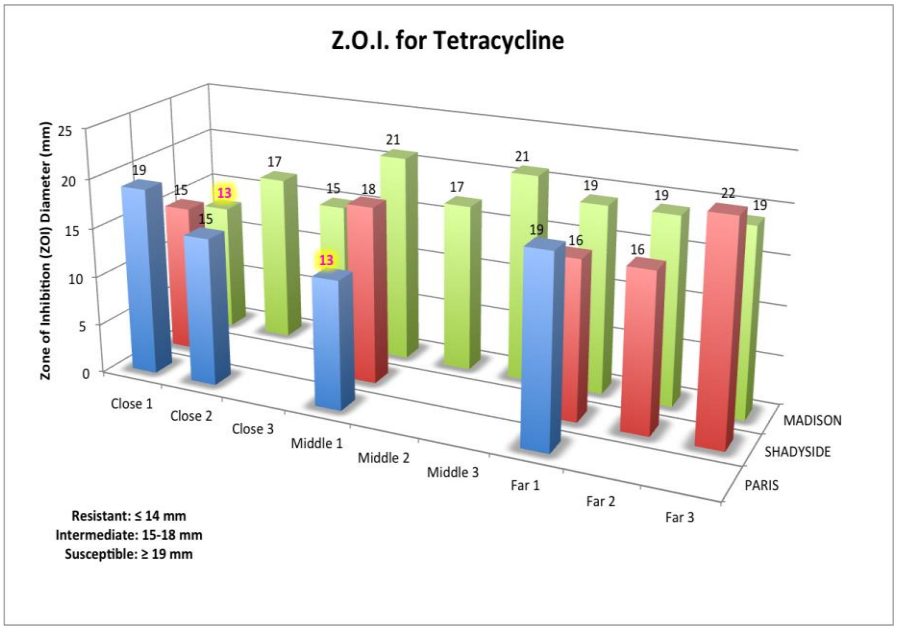

Figure 13. The above, left chart shows the diameter of the Zone of Inhibition in millimeters for each of the colonies tested with Tetracycline. Each number highlighted in yellow represents a colony that is resistant.

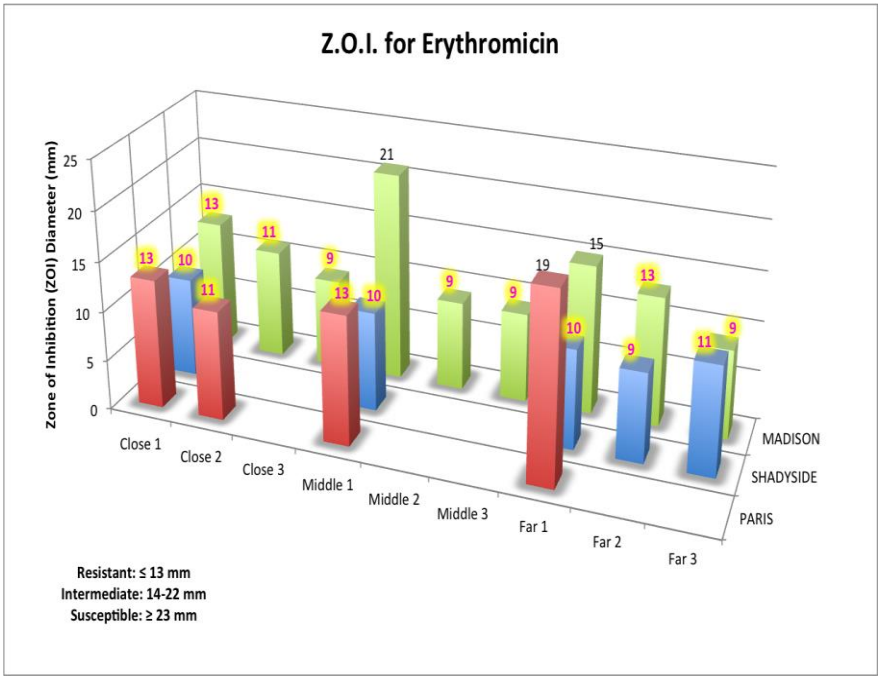

Figure 14. The above, left chart shows the diameter of the Zone of Inhibition in millimeters for each of the colonies tested with Erythromycin. Each number highlighted in yellow represents a colony that is resistant.

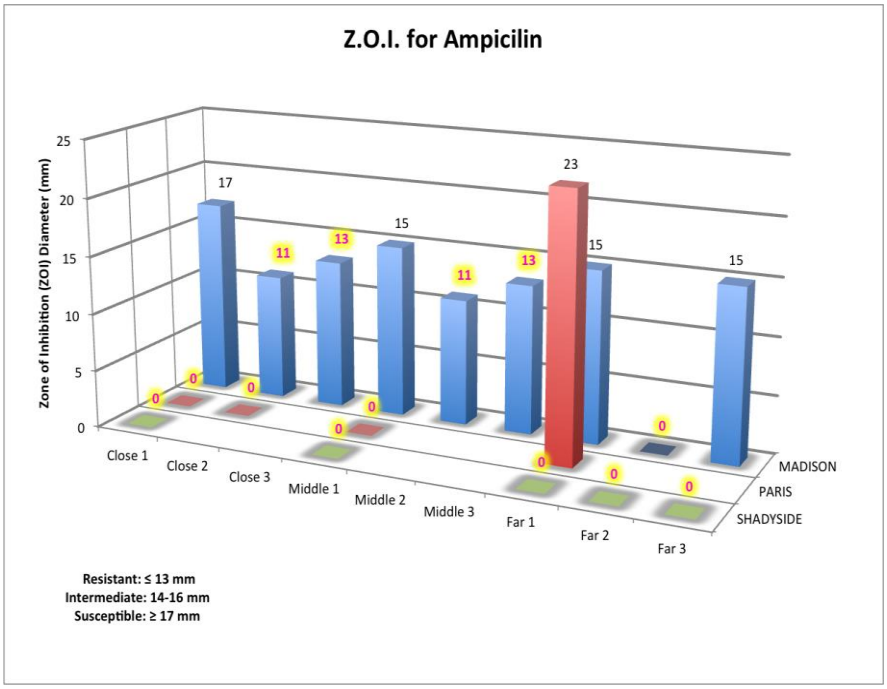

Figure 15. The above, left chart shows the diameter of the Zone of Inhibition in millimeters for each of the colonies tested with Ampicillin. Each number highlighted in yellow represents a colony that is resistant.

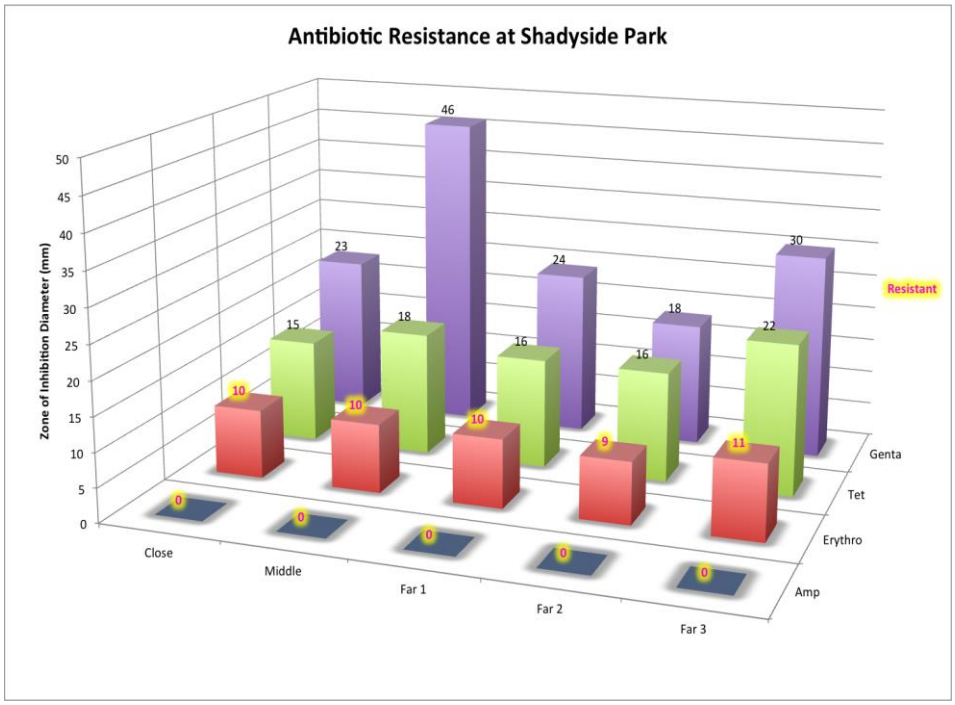

Figure 16. The above graph shows the results of the experiment, specific to Shadyside Park; Resistant colonies are highlighted in yellow.

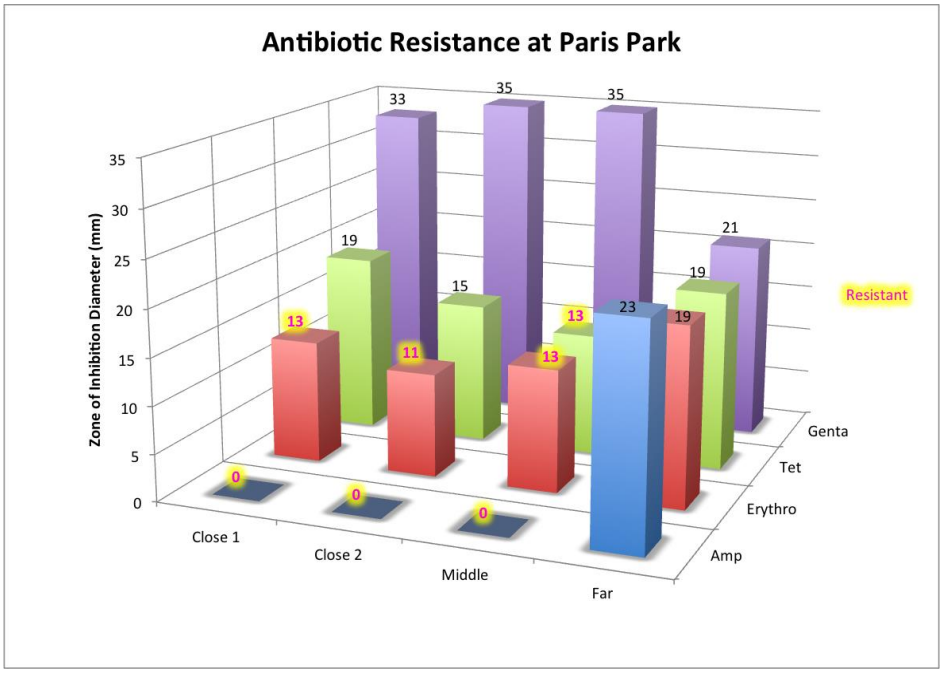

Figure 17. The above graph shows the results of the experiment, specific to Paris Park; Resistant colonies are highlighted in yellow.

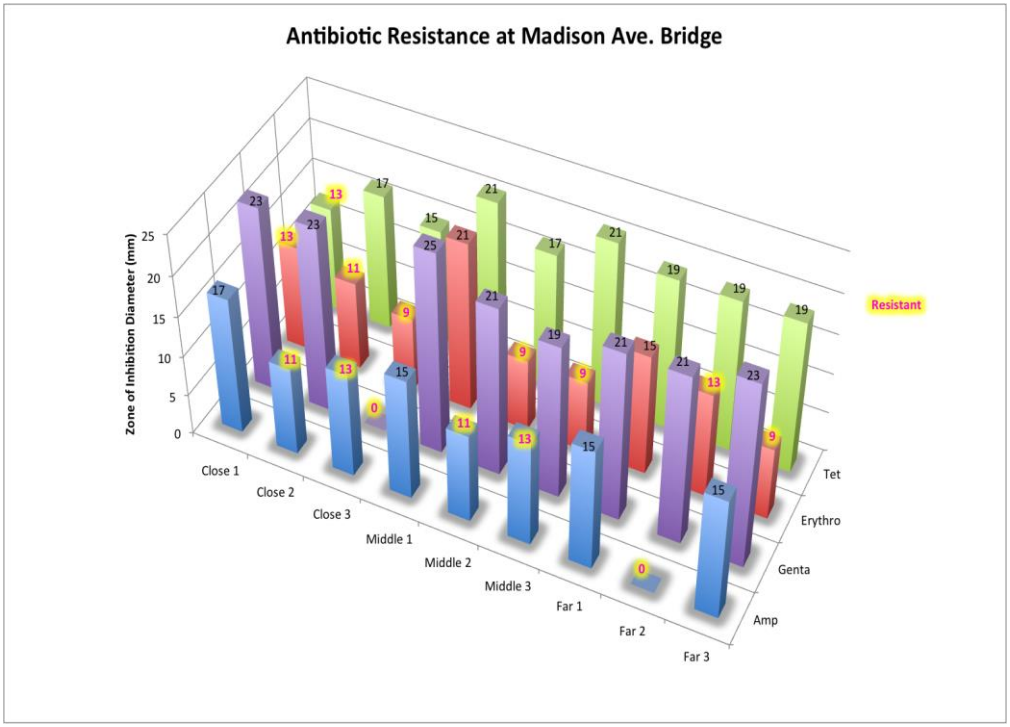

Figure 18. The above graph shows the results of the experiment, specific to Madison Ave. Bridge; Resistant colonies are highlighted in yellow.

Table 1. P-values of Chi Square analysis comparing percentage of resistant strains of *Enterococci* and *E. coli* to four different antibiotics. Comparison was made between two locations, Paris Park and Roosevelt Park. N/A indicates the percentages of the two groups are the same and therefore a p-value cannot be obtained; p-value<0.05 is considered statistically significant.

	Streptomycin	Vancomycin	Ampicillin	Cephalothin
Enterococci	N/A	N/A	0.2733	0.2733
E. coli	0.3613	N/A	0.1709	0.02535

Table 2. T-test results when comparing wet and dry samples at individual locations

Location	**Wet Mean (cfu's)**	**Dry Mean (cfu's)**	**p-Value**
Roosevelt	107,400	9,619	0.0082
Ken-O-Sha	286,700	47,038	0.02716
Shadyside	48,900	3,415	0.0045

Appendix C: Protocols

For Study 1: The existence and distribution of antibiotic resistant *E. coli, Salmonella,* and *Enterococci* in Roosevelt and Paris parks

1. Streak Plate Procedure:
   a) Petri dishes containing agar were labeled for identification.
   b) For each plate, a sterile inoculating loop was used to gently streak designated section taking care not to streak the same area twice.
   c) The process described above was repeated for each designated sections.

2. Standard diameters for Kirby-Bauer Disk by Clinical and Laboratory Standards Institute (2012)

   a) For *Enterobacteriaceae* (*Salmonella* and *E.coli*)

Diameter (mm)	Resistant	Intermediate*	Susceptible
Streptomycin	≤11	12-14	≥ 15
Ampicillin	≤ 13	14-16	≥17
Cephalothin	≤14	15-17	≥18
*Vancomycin (naturally resistant)	-	-	-

*Most Gram-negative bacteria, including *Salmonella* and *E. coli*, are intrinsically resistant to vancomycin because their outer membrane is impermeable to large glycopeptide molecules, of which vancomycin consists.

   b) For *Enterococci*

Diameter (mm)	Resistant	Intermediate**	Susceptible
Streptomycin	≤ 6	7-9	≥ 10
Ampicillin	≤ 16	-	≥17
Cephalothin	≤ 14	-	≥ 15
Vancomycin	≤ 14	15-16	≥ 17

**For this study, diameters in the intermediate category were labeled "Resistant."

For Study 2: Investigation of Enteric Bacterial Concentration and Resistance in Plaster Creek

3. Preparation for triclosan Antibiotic Disk: A dilution of 0.5mg/L of Triclosan was prepared in ethanol. 20 μL of the triclosan dilution was applied to a blank Kirby-Bauer disk. The disk was allowed to dry so that the ethanol evaporated, leaving only the antibiotic on the disk. This disk was used with the other pre-prepared antibiotic disks to measure resistance as detailed later in the procedure.
4. Dry Samples: Dry samples were taken more than 48 hours after the last rainstorm. Three replicates of 45 mL water samples were collected from Roosevelt Park, Ken-O-Sha Park, and Shadyside Park, each in a 50mL plastic centrifuge tube. Once back in the lab, each of these 9 samples

was centrifuged at 4000 rpm for 15 minutes. The excess liquid was decanted, and the pellet was re-suspended in 1 mL of pH neutral phosphate-buffered saline. Then serial dilutions were set up in 2mL Eppendorf tubes, diluting from 10-1 out to 10-3 by transferring 100µL of the original solution into 900µL of pH neutral phosphate-buffered saline, then vortexing and repeating into subsequent tubes. One dilution was plated from each sample (3 replicates at 3 locations, for a total of 9 plates) on EMB agar, using 100µL of the dilution (after vortexing) and spreading the liquid evenly across the plate with a plate spreader. The plates were dried and incubated for 48 hours. After this, the total number of colony forming units (cfu's) was counted as well as the number of unique cfu's on each plate in order to determine the variance in bacterial levels in Plaster Creek. Then 5 different bacterial colonies were selected from the Ken-O-Sha plates (middle reach samples) and isolated the colonies further using a streak plate, which was prepared on Mueller-Hinton plates. These were incubated for 48 hours in a 37ºC incubator. Several loopfuls of each of these pure colonies were transferred into TSB using an inoculating loop. 100µL of this pure culture was pipetted onto Mueller-Hinton plates (2 plates each of 5 different bacteria for a total of 10 plates), and spread the liquid using plate spreaders. While the plates were still wet, the antibiotic disks were placed into the agar using sterilized forceps. Plates were incubated at 37ºC for 48 hours. Then bacterial growth was observed, and the diameter of the clear circle around the antibiotic disk was measured.

5. Wet Samples: The dry sample procedure was repeated using samples taken within 24 hours of a rainstorm with accumulation of 0.5 inches. These samples were diluted from 10-1 to 10-4 to account for the previously documented increase in bacteria levels following a rainstorm.