Analysis of the Impact of Wastewater Treatment Plant Effluent on Antibiotic Resistance in Pennsylvania Stream Microbiomes

Christy Sackett and Dr. Jeffrey Erikson

Biology Department, Messiah University, Mechanicsburg, PA

Abstract

Though antibiotics have served as a powerful tool in humanity's fight against disease, the overuse of antibiotics has caused some bacteria to develop resistances to antibiotics, making these drugs less effective. One means by which bacteria may be exposed to antibiotics and develop resistance is through wastewater treatment plant effluent, which carries antibiotics from human wastes into local streams. We hypothesize that increased levels of antibiotics downstream of treatment plants have selected for increased antibiotic resistance in bacteria living in these downstream regions. As such, when both upstream and downstream samples of bacteria are exposed to the same antibiotic treatments, downstream samples should exhibit higher survival. To test our hypothesis, we sampled bacteria from Dogwood Run and Stony Run in Dillsburg, PA, selecting collection sites from both upstream and downstream of the local wastewater treatment plants. We cultured these bacteria in the lab, classified them based on colony morphology and Gram-stain results, and exposed them to antibiotics through the Kirby-Bauer and replica plating methods. When comparing bacteria only within our classified groups, we found 42 instances of increased downstream resistance and only 23 instances of increased upstream resistance. However, when the resistances between groups were compared with a two-sample T-test, the differences between them were found to be insignificant. These results indicate that the introduction of antibiotics into PA streams has not been sufficient to induce significant levels of antibiotic resistance.

Introduction

- **Wastewater treatment plants are vital for purifying sewage of organic waste and pathogenic bacteria before it is returned to the environment or used again. However, these plants are unregulated in terms of the detection and removal of antibiotics and other pharmaceuticals.**
- **According to a study performed by the United States Geological Survey, many pharmaceuticals, including antibiotics, are present in significantly higher concentrations downstream of wastewater treatment plants in PA streams.[1]**
- **Once the antibiotics are carried into streams by treatment plant effluent, they can begin to act upon the native microbes as a selective agent. The antibiotics can kill off non-resistant bacteria, allowing resistant bacteria to proliferate and to "donate" their antibiotic resistance genes to other microbes.**
- **This increase in the proportion of resistant bacteria would increase the likelihood of humans becoming infected by these more dangerous strains, potentially leading to increased disease mortality.**
- **Few prior studies have focused specifically on the impact of antibiotic presence in effluent on the development of antibiotic resistance in microbiomes. The existing studies have not directly compared the levels of resistance in upstream and downstream bacteria, but instead have focused on the prevalence of resistance genes or on calculating the likelihood that resistance will develop.[2,3]**
- **In this study, we seek to determine if wastewater treatment plants are indeed causing higher rates of antibiotic resistance in stream bacteria and thus are in need of stricter regulations. Our hypothesis is that the microbes downstream of each treatment plant will have higher antibiotic resistance than those upstream of the plant due to the selection pressure introduced by the introduction of antibiotics from the treatment plant effluent.**

Methods

Sample Collection – Bacteria were collected from the Dogwood Run and Stony Run streams in Dillsburg, PA, from sites both upstream and downstream of the wastewater treatment plant on each stream. Bacteria were collected by swabbing rocks on the bottom of the stream to collect well-established colonies.

Culturing and Classification – Bacteria were grown in the lab on nutrient agar and were classified based on their physical characteristics, including colony color, elevation, margin, and form. Unique colonies from each site were isolated and used to make pure broth and streak cultures. Samples from these pure cultures were used to further classify the bacteria based on cell shape and Gram stain results.

Antibiotics – The following antibiotics were tested for resistance: Penicillin, Kanamycin, Ampicillin, Tetracycline, Doxycycline, Novobiocin, and Nitrofurantoin.

Kirby-Bauer Tests – Mueller-Hinton plates were inoculated with 0.2mL of broth culture for each classified bacterium. Once dry, these plates were treated with 3 or 4 BBL™ Sensi-Disc™ Antimicrobial Susceptibility Test Discs (Figure 1).The plates were incubated at room temperature for 24 hours, and the diameter of the zone of inhibition around each disc was measured and compared to the manufacturer's standard for resistance. Tests were performed in duplicate for Dogwood Run samples and triplicate for Stony Run samples.

Replica Plating – LB agar plates were prepared, each containing one of the antibiotics listed above. The plates were uniformly inoculated with the broth cultures using a sterile metal stamp dipped into wells containing the broths. The plates were incubated at room temperature for 72 hours, and resistance was scored based on colony formation (Figure 2). Tests were performed in triplicate.

Statistical Analysis – Averages of the Kirby-Bauer zones of inhibition between upstream and downstream bacteria were compared using a two-sample T test with 95% reliability (Figure 3). Calculations were performed using Minitab 18™.

Results

Figure 1. Example of results from a Kirby-Bauer test. In this method, discs coated in different antibiotics are added to a plate streaked with bacteria to be analyzed. In this example, the bacteria was susceptible to all three antibiotics tested, as evidenced by the formation of a clear zone of inhibition around each disc and no zone around the blank control.

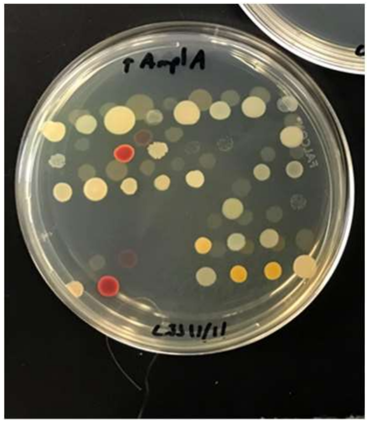

Figure 2. Example of results from a replica plating test. In this method, bacteria were stamped onto an agar plate containing one of the antibiotics under study. The formation of a colony demonstrated the ability of the bacteria to survive and multiply even in the presence of the antibiotic, thus indicating resistance to that antibiotic. In this example, most samples tested were resistant to the Ampicillin used.

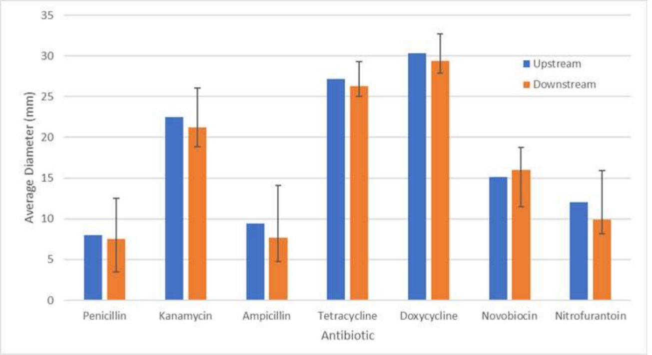

Figure 3. The difference in average antibiotic susceptibility between upstream and downstream bacteria was statistically insignificant. The diameters of the zones of inhibition produced during the Kirby-Bauer tests were averaged for upstream and downstream bacteria, with a smaller zone of inhibition indicating a higher resistance. Although there was a slight trend of increased downstream resistance, none of the differences were statistically significant for any of the antibiotics tested, as determined by a two-sample T test.

Conclusions

- **A variety of bacteria were cultured and classified using large-scale morphology and microscopic features, allowing for comparisons between similar species that might otherwise be overlooked in the averages.**
- **Based on Kirby-Bauer and Replica Plating tests, increased downstream resistance was more common than increased upstream resistance for a given antibiotic and bacteria group. There were 42 instances in which downstream bacteria of a particular type were more resistant to an antibiotic, but only 23 instances in which an upstream bacteria was more resistant.**
- **When comparing overall averages, the downstream bacteria initially appeared to be more resistant than upstream bacteria for all antibiotics except novobiocin. However, none of the differences between upstream and downstream resistance were statistically significant.**
- **Based on our results, antibiotic introduction through wastewater treatment plant effluent does not appear to be inducing a significant increase in antibiotic resistance in downstream bacteria.**
- **The lack of downstream effects of antibiotic introduction may be due to insufficient antibiotic concentrations to induce a selection pressure. Alternatively, the similarity between upstream and downstream resistance may be due to upstream introduction of antibiotics by residential septic tanks.**

Acknowledgments

We thank the Messiah University Biology Department for providing funding and equipment for this experiment. We thank Dr. Jesse Kleingardner for supplying the kanamycin used in this experiment.

References

[1] **Loper CA, Crawford JK, Otto KL, Manning RL, Meyer MT, *et al.*** *Concentrations of Selected Pharmaceuticals and Antibiotics in South-Central Pennsylvania Waters, March through September 2006*. Reston, VA: U.S. Geological Survey. https://pubs.usgs.gov/ds/300/ (2007, accessed 9 March 2020).

[2] **Lambirth K, Tsilimigras M, Lulla A, Johnson J, Al-Shaer A, *et al.*** Microbial Community Composition and Antibiotic Resistance Genes within a North Carolina Urban Water System. *Water* 2018;10:1539.

[3] **Kairigo P, Ngumba E, Sundberg L-R, Gachanja A, Tuhkanen T**. Occurrence of antibiotics and risk of antibiotic resistance evolution in selected Kenyan wastewaters, surface waters and sediments. *Science of The Total Environment* 2020;720:137580.