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Describing the Impact of the Sales of Antibiotics on the Environment and in Population Health

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DESCRIBING THE IMPACT OF THE SALES OF ANTIBIOTICS ON THE ENVIRONMENT AND IN POPULATION HEALTH

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DEDICATION

The mental health of a doctorate student will constantly change throughout a program of study. It will affect directly the creativity, dedication, and ambition used when developing the research. I want to mention this because, in my experience, there were several moments where I could not find value in what I was doing. It translated into a diminished value of myself. In those moments, I felt I did not have the ability to filter many emotions and focus on the goals I had initially set for my study. I felt I did not have the mental capacity to obtain the doctorate degree.

There were many friends that supported me throughout this endeavor. Those friends were the ones that made me believe that it is not only knowledge that is valuable when obtaining this kind of degree. I want to thank that friend, that when I was told that my aspirations were irrelevant, they made me see that it was relevant if I believed in it. Also, a big thank you to those friends that allowed me to take as many steps as I needed, in the direction I thought it was right, until I was ready to continue. Special thank you to that friend that picked me up and carried me because I did not think I was capable of taking any more steps, and also those friends that sacrificed many hours of sleep just to give me reassurance in myself. I also want to thank my dad and my sister; they have always showed me their unconditional support.

My Mom never doubted in my abilities, her encouragement was always love. I want to dedicate this to her.

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ABSTRACT

The role of antibiotics in human health is of significant importance in the advancement of the medical field. It has contributed to an improvement of the quality of life and the age expectancy of the population. Antibiotic resistance is an expected consequence of the use of antibiotics. Bacteria have developed biological mechanisms that would help them become resistant to antibiotics. The uncontrolled use of antibiotics is significantly contributing to the ability of bacteria to become resistant to those antibiotics. This study, using the model of the Socio-Ecological Coupling of Antibiotic Resistance, explains how social and environmental factors interact and impact the social and natural cycle of antibiotic resistance. Using spatial analysis, the study describes how antibiotic sales in combination with other demographic factors contribute to the appearance of antibiotic sales hot spots. The expectation of antibiotic resistance in these hot spots is higher and the urgency for intervention with antibiotic stewardship programs at the community level is necessary. Experimentally, the study demonstrates how wastewater treatment plants, being the receptacle for most antibiotics consumed, can become a source of exposure to antibiotic resistant genes through bioaerosols. Lastly, using epidemiological methods, the study describes temporal trends of antibiotic sales and how these can be used as an indicator for population health.

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INTRODUCTION

The population of South Carolina has a higher morbidity and mortality rate than the United States average. In each of the top ten (10) conditions causing death compiled by the Center of Disease Control (CDC), the 2017South Carolina rates of deaths per person were above the national average. In most of the CDC categories, South Carolina's rate ranked in the top 16 states. (Table 1.1).

Table 1.1. SC Leading Causes of Death, 2017.

Condition	Deaths	Rate*	State Rank	U.S. Rate*
1. Heart Disease	10,418	172	16th	165
2. Cancer	10,356	162.7	14th	152.5
3. Accidents	3,147	60.2	13th	49.4
4. Chronic Lower Respiratory Diseases	2,983	47.9	12th	40.9
5. Stroke	2,691	44.9	4th	37.6
6. Alzheimer's disease	2,549	44.9	6th	31
7. Diabetes	1,535	24.5	12th	21.5
8. Kidney Disease	950	15.5	16th	13
9. Septicemia	884	14.5	10th	10.6
10. Suicide	838	16.3	25th (tie)	14

*Age Adjusted

Source: CDC/National Center for Health Statistics.
https://www.cdc.gov/nchs/pressroom/states/southcarolina/southcarolina.htm

The rate of prescriptions of antibiotics that were sold in South Carolina in 2017 was also higher than the national average: South Carolina had 881

prescriptions sold per 1000 population, while the national rate was 794 per 1000 population (Table 1.2).

Table 1.2. Estimated Antibiotic Prescription Sales in the United States in 2017.

State	Prescriptions per 1000 Population
Alabama	1117
Alaska	451
Arizona	683
Arkansas	1057
California	526
Colorado	525
Connecticut	790
Delaware	820
Florida	784
Georgia	898
Hawaii	572
Idaho	626
Illinois	781
Indiana	891
Iowa	915
Kansas	885
Kentucky	1176
Louisiana	1140
Maine	658
Maryland	720
Massachusetts	701
Michigan	857
Minnesota	627
Mississippi	1152
Missouri	854
Montana	606
Nebraska	944
Nevada	659
New Hampshire	675
New Jersey	848
New Mexico	642
New York	859
North Carolina	834

North Dakota	742
Ohio	903
Oklahoma	858
Oregon	488
Pennsylvania	847
Rhode Island	832
South Carolina	**881**
South Dakota	833
Tennessee	1083
Texas	805
Utah	675
Vermont	601
Virginia	747
Washington	516
West Virginia	1228
Wisconsin	663
Wyoming	718
United States Average	**794**

Source: The Center for Disease Dynamics, Economics & Policy. Resistance Map: Use of All Antibiotics 2017. 2022. resistancemap.cddep.org/CountryPageSub.php?countryId=38&country=United+States. Accessed: January 2022.

States that experienced a higher rate of antibiotic prescriptions sold, including South Carolina, also share (i) a higher rate of morbidity and mortality compared to the national average, (ii) an aging population, (iii) less restrictions on the prescription of antibiotics, and (iv) fewer antibiotic stewardship programs (County Health Rankings and The Center for Disease Dynamics Economics and Policy (CDDEP)). Although antibiotic use has not previously been used as an indicator for health status in the population, the following study shows that it can be valuable in environmental surveillance, provide an approximate measure for health behavior, and determine the effectiveness of stewardship programs and policy.

With a total population of 5,024,369 in 2017 (United States Census Bureau, 2022), most of the 46 counties in South Carolina could be considered rural counties with half of the population concentrated in seven (7) counties: Greenville (506,837), Richland (411,592), Charleston (401,438), Horry (333,268), Spartanburg (306,854), Lexington (290,642), and York (266,439) counties. Major hospitals and medical facilities, as well as major water treatment plants and wastewater treatment plants, are also situated in these main urban areas. The geography of the State varies from piedmont in the west to an almost 3,000-mile tidal coastline to the east (NOAA, 2019). Two of the major economic activities in South Carolina are agricultural and industrial, with a significant amount of concentrated animal and farming operations in the north-east of the State. According to the Department of Health and Environmental Control, thirty-three percent (33%) of the population in South Carolina live in rural areas and derive their livelihood from agricultural practices. (South Carolina Department of Health and Environmental Control, 2022). Activities for this population include subsistence fishing, use of unregulated drinking water systems, and active use of recreational waters (Burch et al., 2014). These activities present a higher risk of exposure to environmental pollutants that include emerging infectious diseases agents such as antibiotic resistant microorganisms.

Antibiotic use has increased significantly since their discovery in 1928. Early use of antibiotics occurred only in hospitals and medical facilities, but with time, research, and familiarity, antibiotics were prescribed to be used by individuals at home. Currently, antibiotic prescriptions sold in outpatient settings

and used by individuals comprise more than 70% of the total antibiotic use for human health purposes (Sriram, 2021). The dataset utilized for the research in this study includes the total antibiotic prescriptions sold in outpatient settings from 1999 to 2017 per county in South Carolina. Table 1.3 describes an estimation of the rate per 1000 population of the total antibiotics sold per county in South Carolina during 2017.

Table 1.3. Rate per 1000 population of Antibiotic Prescription Sales per County in South Carolina in 2017

County	Prescription sales per 1000 population in 2017
Abbeville	534
Aiken	663
Allendale	808
Anderson	794
Bamberg	561
Barnwell	772
Beaufort	726
Berkeley	267
Calhoun	78
Charleston	1436
Cherokee	650
Chester	693
Chesterfield	403
Clarendon	625
Colleton	844
Darlington	1124
Dillon	945
Dorchester	709
Edgefield	224
Fairfield	347
Florence	1401
Georgetown	1264
Greenville	1185
Greenwood	1299
Hampton	645
Horry	954
Jasper	951

Kershaw	724
Lancaster	601
Laurens	566
Lee	205
Lexington	807
Marion	916
Marlboro	501
McCormick	198
Newberry	550
Oconee	718
Orangeburg	743
Pickens	721
Richland	1005
Saluda	333
Spartanburg	962
Sumter	843
Union	534
Williamsburg	532
York	632

Source: CDDEP.

Clinically, there are different factors that lead to antibiotic use. The main purpose is to tackle bacterial infections. Most common infections are respiratory infections, urinary tract infections, and sexually transmitted infections. Patients with chronic health conditions, that have undergone an organ transplant, or will undergo a major surgery also depend on the use of antibiotics. During these situations, antibiotics could be may be used preventively as a bacterial infection for any of these patients would be fatal. Additionally, bacterial infections have similar symptoms, and can be easily misinterpreted, as viral infections. In practice, physicians do not screen every patient to determine whether certain symptoms are caused by a bacterial or viral infection, and because bacterial infections are considered significantly more severe to viral infections, physicians prefer to prescribe antibiotics in most situations.

All of these practices for the use of antibiotics have accelerated the pace of the development of antibiotic resistant bacteria (ARB). The familiarity and the wide use of broad-spectrum antibiotics have increased the number of bacterial infections that are no longer susceptible to many antibiotics. The major consequence of the uncontrolled and indiscriminate use of antibiotics is ARB.

The purpose of this research is to analyze the geographical and temporal trends of antibiotic use in South Carolina. Chapter 3 describes a spatial analysis of antibiotic use and the effect of population in South Carolina from 1999 to 2017. Chapter 4 is an experimental study of the aerosolization of ARB and the probable dispersion of antibiotic resistance genes from wastewater treatment plants. Lastly Chapter 5 is a seasonal and longitudinal analysis of antibiotic use and its possible impact on overall health in the population.

CHAPTER 2

BACKGROUND AND SIGNIFICANCE

The use of antibiotics was one of the major breakthroughs in the twentieth century. It revolutionized the field of medicine and was one of the major factors in decreasing the mortality from communicable diseases (IOM, 2010). The ability to treat and cure infectious diseases allowed the health field to advance research in organ transplants, surgical procedures and public health surveillance. The control of bacterial pathogens with antibiotics was also introduced in the agriculture and the animal production industries (Drexler, 2010). Antibiotic use shifted farming practices by allowing higher yields in crops and less disease in animals with minimum loss in smaller production areas (NRC, 1980). Although antibiotics became a solution for a critical problem, the indiscriminate use that followed is now thought to have accelerated the rate at which bacteria are developing resistance (NRC, 2003; Colomer 2011; Spicknall, Foxman, Marrs, & Eisenberg, 2013).

When bacteria are exposed to minimum inhibitory concentrations (MIC) of antibiotics, a process similar to natural selection occurs (Drexler, 2010; Nesme et al., 2014). Bacteria genetically adapt to the presence of antibiotics by acquiring a method of resistance such as DNA point mutations and/or a horizontal gene transfer of resistance genes (Sidrach-Cardona, Hijosa-Valsero, Marti, Balcázar,

& Becares, 2014). Those surviving bacteria, that have the antibiotic resistant genes (ARG), will have the capacity to pass along or exchange those ARG. This process of acquiring resistance occurs to a higher extent in the natural environment, but also through incorrect use in hospitals, food products, animals, and the human body (Colomer, 2011; Salipante, 2013). For example, the amount and variety of antibiotics deposited in the environment through wastewater treatment plants and farming operations creates stressful conditions for bacteria. The low concentration of antibiotics will likely not eliminate the bacterial community completely, but will facilitate their adaptation to the new environmental conditions in the presence of the antibiotics (Summers, 2002). The large amount of antibiotics in the environment has accelerated a process that would have taken millions of years with natural occurring antibiotics, to one that has taken only decades or even years.

The rate of morbidity and mortality due to antibiotic resistance is increasing every year. The World Health Organization estimates that currently 700,000 people die from antibiotic resistant infections a year worldwide (World Health Organization, 2019). It is predicted that if current conditions continue, the global death toll per year will be close to 10 million. Clinics and hospitals are now experiencing challenges when treating infections that did not use to be life threatening. Broad spectrum antibiotics are not as effective as when they were first developed. Narrow spectrum antibiotics, which are significantly more expensive, are now being used more frequently. A major concern is that bacteria will acquire resistance to narrow spectrum antibiotics because of their current

wide use. Resistance to antibiotics is moving faster than the pharmaceutical development pipeline.

Medical facilities present ideal conditions for bacteria to acquire antibiotic resistance, such as: 1) presence of a large number of antibiotics; 2) presence of immune deficient individuals; 3) increasing proximity and interaction between pathogens; and 4) not effective sanitizing behaviors. (Struelens, 1998; Agency for Healthcare Research and Quality, 2018). However, research has also demonstrated that exposure to ARB is not only occurring in hospital settings, but also at the community level (CDC, 2015, Colomer, 2011). But while hospital acquired infections are monitored closely and specific stewardship programs have been developed to address them, the same cannot be said about community acquired infections (Goosens, 1998; Wunderink, 2016).

The probability of an outbreak from an ARB infection in the community could have significant consequences. There is no current clinical solution to treat several infections caused by ARB, and if ARB growth is not contained, infectious diseases could once again become a major cause of mortality in the developed world and could become an even larger burden in low-income populations.

One mechanism of control against ARB is antibiotic stewardship programs. Most of these programs promote better screening mechanisms of infectious diseases in hospitals to provide accurate diagnoses, targeted and appropriate use of antibiotics when they are needed, collection of unused antibiotics, and public education on the use of antibiotics. A second mechanism is environmental surveillance. Continuous monitoring of the natural environment

and recognizing any changes to ecosystems services can be useful in identifying trends to forecast the appearance of an outbreak, or in the case of this study, to learn when any changes could be setting off an alarm (i.e., increase in antibiotic sales can represent premature mortality in a population).

An important tool to help with the identification of key locations for environmental surveillance is spatial analysis. With Geographical Informational Systems (GIS), it is possible to identify changes in diseases patterns within population in a geographical area and between geographical locations. These patterns cannot only be described as a snapshot, but also as a temporal trend. In conjunction with the concept of socio-ecological networks described in Chapter 4, particularly the socio-ecological coupling of antibiotic resistance (Figure 2.1), these tools can help identify the hot-spots for antibiotic resistance.

Wastewater treatment plants (WWTPs) is one hot-spot location. WWTPs can discharge into the environment low concentrations of antibiotics as well as antibiotic resistant genes from living and dead microbial cells, causing many ecosystems connected to the WWTP to potentially serve as reservoirs of antibiotic resistant microbes. These dynamics could cause a WWTP to be an augmenter of ARB.

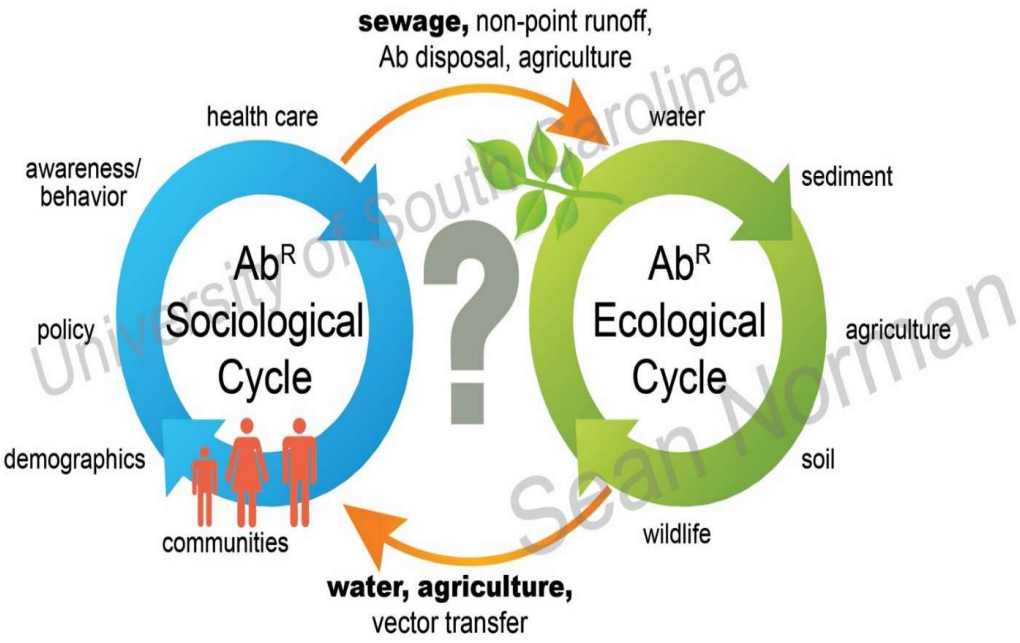


Figure 2.1. Socio-Ecological Coupling of Antibiotic Resistance (Gaviria-Figueroa, 2019).

Socio-Ecological Coupling of Antibiotic Resistance (SECAR) is a representation of the interaction between social factors and environmental factors. The Sociological cycle includes human behaviors that have an impact on health and use of antibiotics. The Ecological cycle shows how antibiotics can be maintained and become part of the environment. In one direction, human activity is impacting the natural environment by the disposal of antibiotics through sewage, waste, and use in agriculture. In return, humans are experiencing environmental changes and an increase rate of disease from infections that are resistant to most or all known antibiotics. There is a wide gap in the knowledge of how the two cycles are connected. A hypothesis of the research described in this study is that antibiotic sales and usage are the engines making the cycles flow.

Human activity is increasing selective pressure on microorganisms, which is conducive for the emergence or re-emergence of pathogenic microorganisms. Identifying the hot-spots where there is a higher risk for antibiotic resistant gene exchange might contribute to preventing an ARB outbreak and can become an important component of antibiotic stewardship programs. The research in this study shows how antibiotic sales and usage can be used to help identify these hot-spots.

CHAPTER 3:

CLUSTER ANALYSIS OF THE AMOUNT OF ANTIBIOTICS SOLD PER COUNTY IN SOUTH CAROLINA DURING 1999 -2017

Studying a geographic region may contribute to health research when data is available about its population and a baseline can be defined so it can be compared through time. Geographical studies, specially those involving larger regions (i.e. a state) can be easily adapted to both urban and rural environments, which is important as factors related to consumption may vary significantly with population size and density, and multiple study sites may be necessary to account for this dynamic (Lancet, 2015). Identification of spatial and temporal trends is important to understand the epidemiology of antibiotic resistance. The identification of regions where consumption is high is necessary to determine if there is a higher probability for resistant infections to occur in those areas. For example, identification of geographical hotspots can provide a baseline for implementation of antibiotic stewardship programs and locations to establish environmental surveillance (Van Boeckel, 2014).

The misuse and over use of antibiotics is the leading cause of occurrence of antibiotic resistance at the clinical setting, at the community level, and in the environment (Ventola, 2015). Parallel to increasing the selection for resistance, antibiotics also lose their effectiveness against pathogens. A known tendency is

that antibiotics are relied upon more heavily in areas with a weak public health infrastructure, and antibiotic resistance prevalence is higher when there is lack of sanitation (Aslam, et.al. 2018). The identification of clusters or hot-spots of high antibiotic use is an important finding for implementation of stewardship programs. The following spatial analysis describes the relationship between antibiotic use and populations changes within and between counties in South Carolina from 1999 to 2017. Later in Chapter 5 the statistical analysis will go further in describing if antibiotic use and population changes can be an indicator for premature death.

Methods

The data for this study was provided by the Center for Disease Dynamics, Economics & Policy (CDDEP) through direct agreement with the University of South Carolina and the Molecular Microbial Ecology Lab at the Arnold School of Public Health. The data was collected and originally owned by IMS Health IQVIA MIDAS (IMS Health, Danbury, CT, USA). With use of national sample surveys done by pharmaceutical sales distribution channels (i.e., from manufacturer to wholesaler to retailer), this database estimates antibiotic consumption from the volume of antibiotics sold in retail and hospital pharmacies. It includes more than 70% of the total antibiotics sold in South Carolina. It does not include antibiotics that were prescribed and using in in-hospital patients, and not all used in animals. In each sector, data are collected regularly to estimate direct sales from antibiotic drug manufacturers and indirect sales from wholesalers. The sales estimates from this sample are projected with the use of an algorithm developed

by IMS Health to approximate total volumes for sales and consumption. The algorithm uses regional factors and sectorial-specific and distribution-channel-specific factors to project national estimates of antibiotic consumption. However, precise details of the algorithm are withheld for proprietary reasons (CDDEP, 2017). The variables included in the analysis were total antibiotic prescription sales, total quantity of antibiotics, Defined Daily Dose value, and antibiotic class. All variables were stratified by month, year, and county from 1999 to 2017.

Spatial analysis

The data was analyzed using ArcGIS Pro Version 2.5 from Esri Inc. Maps were created to show the differences and variations of the variables from the data set in a temporal and geographic manner. The different colors were used to symbolize differences between population data (Census, 2022) and antibiotic use from CDDEP data in raster data publicly available. A cluster analysis using the spatial statistics time series tool provided in ArcGIS Pro was used to create the map highlighting the hot spots of antibiotic use.

Results

The map using the rate per capita of antibiotic prescription sales in each county in South Carolina (Figure 3.1) describes the significant variance of antibiotic prescription sales between counties in South Carolina. It shows that three (3) of the seven (7) most populated counties mentioned in the Introduction (Charleston, Florence, and Greenville) are more than two (2) standard deviations above the mean of prescription sales in the State, another three (3) of those

seven (7) are 1.5 standard deviations above the mean (Richland, Horry, and Spartanburg), and one (1) of those seven (7) is only 0.5 standard deviations above the mean (Horry). Most importantly, all of those seven counties are above the mean prescription sales of the State. Of the remaining 39 counties, four (4) are significantly below the mean of the State, by more than 1.5 standard deviations, nine (9) are below the mean by -1.5 and -0.5 standard deviations, the majority of the counties, twenty-one (21) counties, are below or above the mean by 0.5 standard deviations, and the last five (5) counties are above the mean by 1.5 standard deviations.

By looking at those rates in the map, some clusters are evident. There seems to be a concentration of sales in the eastern and north eastern part of the State and a smaller cluster towards the north, north western part of the State. There is a strong correlation between the two variables (number of prescription and quantity of the antibiotics), showing very little difference between running the analysis with quantity of antibiotics and without. As a result, it is concluded that only using prescription sales and population numbers describes the trends.

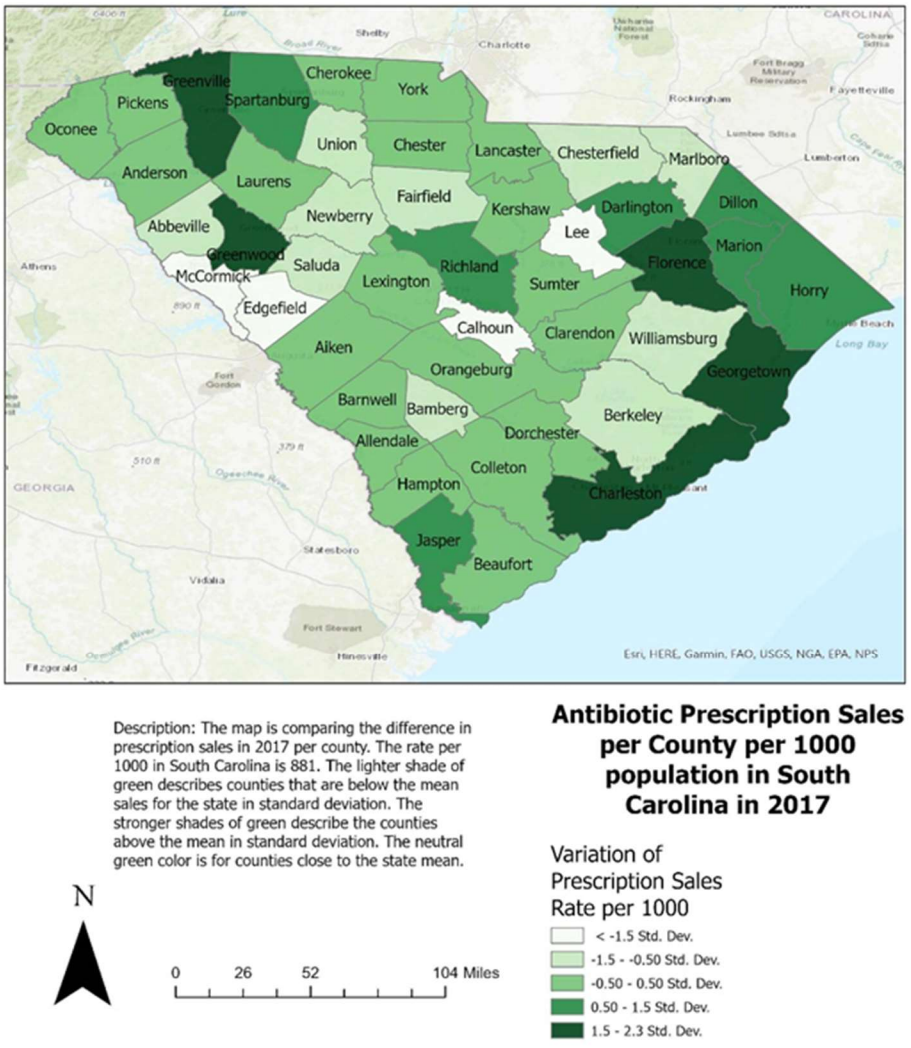


Figure 3.1: Map of South Carolina counties describing the rate of antibiotic prescription sales per 1000 using the standard deviation.

Population Trends in South Carolina

An important factor to look at is at the population of the counties in South Carolina: not only at the current population numbers, but how those

numbers have changed in the last two decades. As a whole, the State has seen an increase in total population of almost 77% in the past 20 years. The State population in 1999 was 3,885,736 and in 2017 it was 5,024,369. In comparison with the grown of other states during that time, it would appear that South Carolina's population has increased significantly and that, subsequently antibiotic sales have increased.

However, in reviewing the data at a granular level, it is apparent that conclusion is not so straightforward. By looking at the data in the map about population change (Figure 3.2), there are significant differences between counties in terms of population growth. The population increase in the last two decades seems to be concentrated in less than half of the counties (seventeen (17) counties) and in three (3) major areas: north west, middle and southeast. Those areas correspond to the major urban areas in the State. One thing to consider is that the biggest loss for any county was not more than 6,000 people (Williamsburg's population in 1999 was 36,840 and in 2017 it was 31,133). The county with the biggest gain in population was Horry County with 154,718 individuals (the population in 1999 was 178,550 and in 2017 it was 333,268).

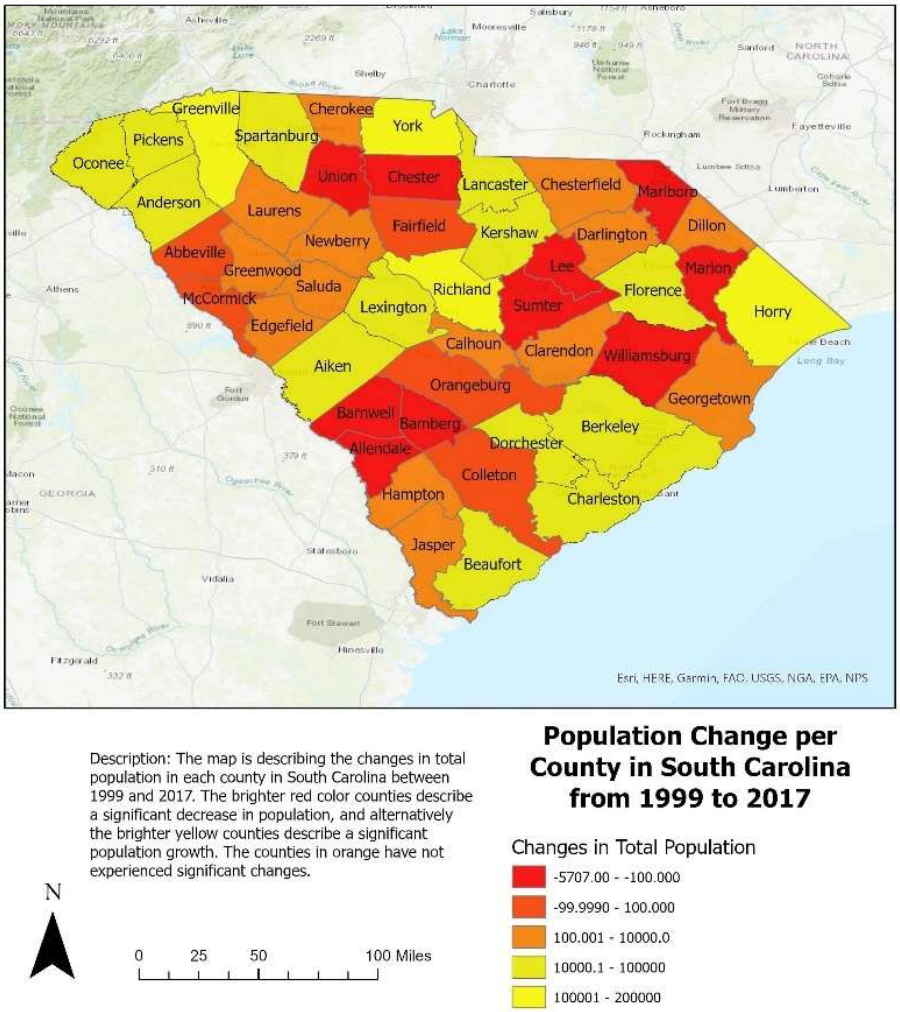


Figure 3.2. Difference in total population per county in South Carolina between 1999 and 2017.

Antibiotic Prescription Sales Differences

Using spatial analysis with GIS software permits the comparison of how different variables behave simultaneously in space. It gives an extra dimension to the statistical analysis. In the analysis of antibiotic sales in the last eighteen (18)

years, counties can be broken in half: twenty-two (22) show a decrease in antibiotic sales, two (2) have remained within the same numbers, and the remaining twenty-two (22) show an increase in antibiotic sales.

If we compare the map about population differences between counties from 1999 to 2017 (Figure 3.2) with the map about antibiotic sales differences during the same time (Figure 3.3), there is a distinct variation in color for many of the counties. For most of the counties that increased in population during those two decades, Figure 3.3 shows that antibiotic prescription sales also increased (although not for all of them). The same cannot be said for the counties that decreased in population or remained with similar population numbers. For those counties, there is a variation between whether there was an increase and decrease in antibiotic prescription sales. The biggest differences between the two maps can be seen between the west, north-west counties and eastern counties. It could be interesting to research further how the agricultural sector has influenced these two areas both in terms of population changes and in antibiotic sales.

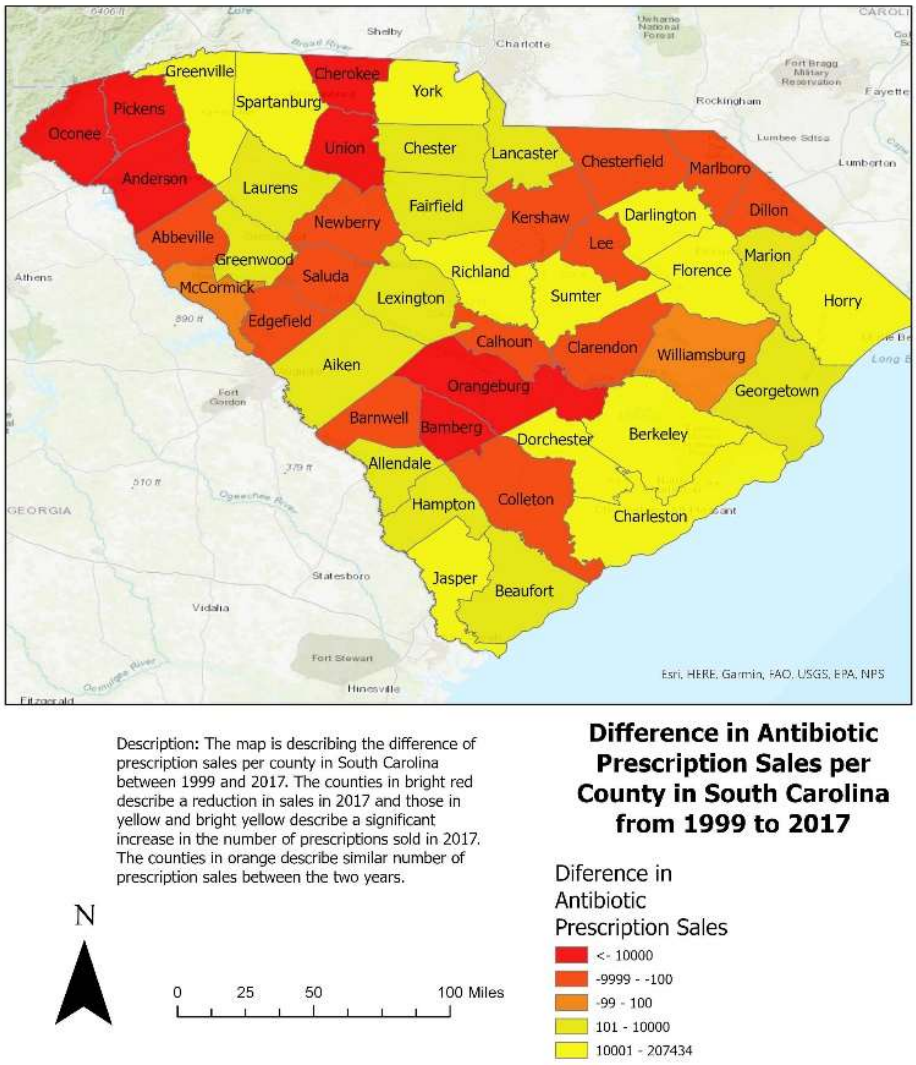


Figure 3.3. Difference in total antibiotic prescription sales per county in South Carolina between 1999 and 2017.

Time Series Analysis

The result of the Emerging Hot Spot Analysis (ArcGis Pro, 2021) shows how using spatial analysis helps incorporate spatial as well as time related

factors in multi-level model analysis. The analysis takes into consideration time association, geographical proximity influences, and subject related factors.

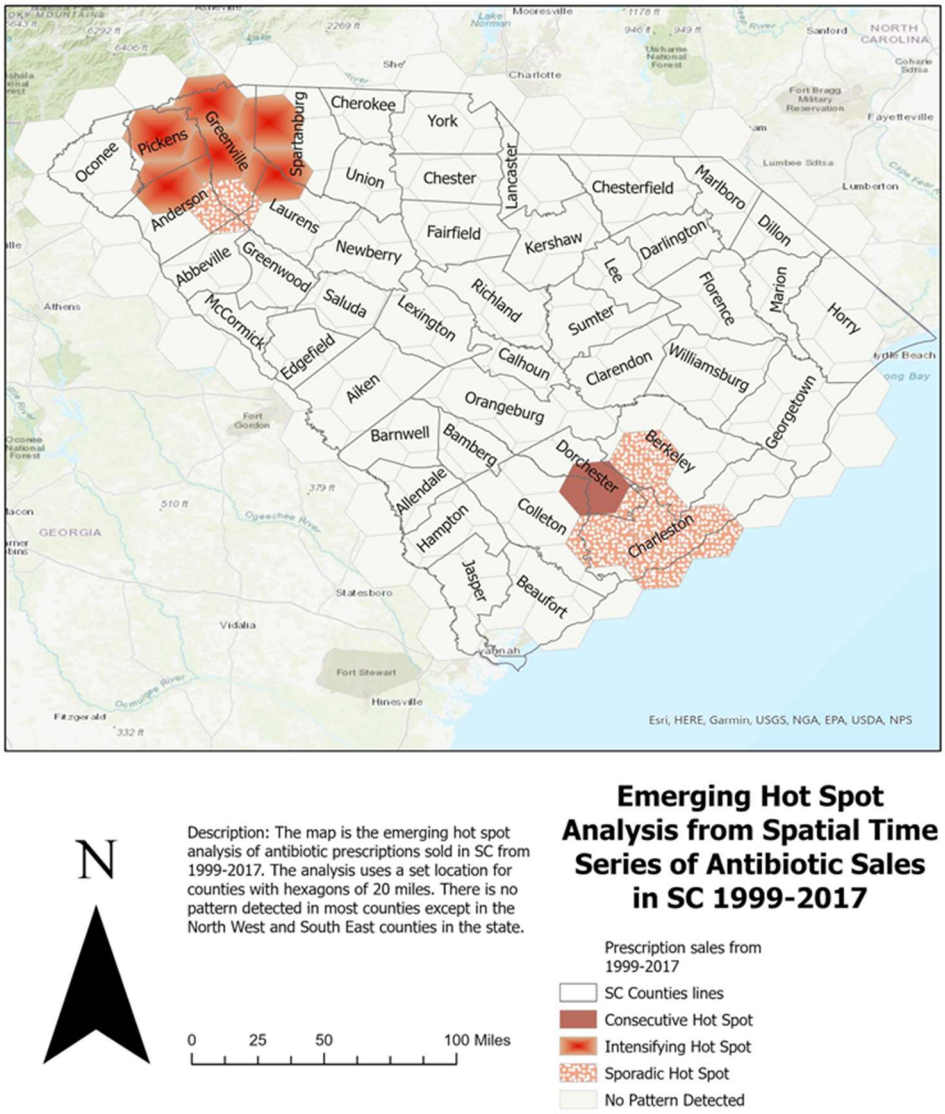


Figure 3.4. Emerging Hot Spot Analysis in South Carolina from Antibiotic Prescription Sales Data from 1999 to 2017.

The geographical locations that appear with different shades of red in the map in Figure 3.4, describe different trends for the antibiotic prescription sales identified. Other factors such as age of the population, immigration, and other demographic factors need to be looked closer. Interventions on antibiotic use are necessary for the two locations, but the difference in trends imply that a different approach is necessary.

Conclusion

With these series of maps, we can conclude that population is a decisive factor in determining the status of antibiotic prescription sales. Changes within short periods of time can provide a general image, but to avoid an ecological fallacy, the time period in this study is from data from significant period of time and at a granular geographical spectrum. Advances in GIS software allow a review of the data in three dimensions (data, place, and time). The cluster analysis is not only of a snapshot of the State at a particular time, but also describes the trends for the State. Environmental surveillance can be narrowed down to not only locations that were expected (dense urban areas) to have high antibiotic usage, but also to locations that are showing the larger differences in population and antibiotic sales in the last two decades. These locations could see bigger changes in the environment and higher antibiotic loading (Browne, et al, 2021).

Something that has not been included and could help give a clearer picture is adjusting for age in the population, although this has not been deterministic in the literature. It is expected that older individuals will be

consuming more antibiotics, but it mostly occurs at the hospital setting (Browne, et al, 2021). Research in children shows that the consumption of antibiotics is very high and because of the nature of the infections, most antibiotics are prescribed in outpatient settings (Browne, et al, 2021). Research has also shown that working adults are exposed to higher degrees of bacterial and viral infections and over the counter antibiotics seemed to be over prescribed for this age group (Reynolds, 2016). Adjusting for age could help analyze how the age of a population affects antibiotic usage in the various South Carolina counties (Browne, et. Al, 2021).

In this Chapter, it has been shown that there is an association between population density, geographical location, and antibiotic sales. In an attempt to further the research and narrow the gap between the connection between antibiotic sales and overall population health, in Chapter 5, the longitudinal study describes how antibiotic sales can be linked to increase or decrease to premature death. Chapter 4 is an experimental case scenario of how WWTP can impact neighboring areas and explain how antibiotic loading can affect dense populations in higher proportion. It is also an example of proposed environmental surveillance that could help control the dispersion of antibiotic resistant genes and consequently antibiotic resistant gene exchange.

CHAPTER 4:

EMISSION AND DISPERSAL OF ANTIBIOTIC RESISTANCE GENES THROUGH BIOAEROSOLS GENERATED DURING THE TREATMENTS OF MUNICIPAL SEWAGE

Without the implementation of better control measures, it is estimated that the number of yearly global deaths related to antibiotic resistant infections will continue increasing, becoming widespread across many economic and public health sectors (O'Neill 2014; Pruden 2014; Berendonk et al., 2015). In an effort to reduce the spread of ARB, it is important to understand the socio-ecological coupling of resistance (i.e., how antibiotics and ARB within social systems transmit to and cycle within environmental systems) and routes of potential human exposure within these coupled systems.

In urban settings of developed and developing nations, wastewater treatment plants (WWTPs) act as socio-ecological couplers through the concentration, treatment, and subsequent environmental release of sewage collected from surrounding communities. Given that antibiotics, ARB, and, ARGs have been observed in municipal sewage, WWTPs are often considered a significant reservoir of antibiotic resistance (Uyaguari et al., 2011; Rizzo et al., 2013; Yang et al., 2014; Mao et al., 2015; Xu et al., 2015; Zhang et al., 2015; Guo et al., 2017). Additionally, it has been shown that the abundance of

antibiotic resistance genes may be higher in WWTP effluent than in raw sewage, suggesting that the treatment process could be selecting for more resistant bacteria. Studies have further shown that ARGs can be transferred from WWTPs into surrounding ecosystems through the release of treated effluent (Czekalski et al., 2012; Xu et al., 2015, Zhang et al., 2015; Chu et al., 2018). While studies have identified the release of ARB/ARGs in treated sewage, little is known about potential dispersal through wastewater bioaerosol (i.e., airborne particles of biological origin) emissions.

It is generally accepted that exposure of wastewater workers to bioaerosols carries a risk of negative health outcomes. This thought is based on the fact that sewage is known to contain a range of potential pathogens (Feachem et al., 1983; Rose 1986; Shuval et al., 1986; Wéry et al., 2008) and that some studies have suggested a correlation between exposure of WWTP bioaerosols and a range of respiratory and gastrointestinal symptoms (Rylander et al., 1999; Douwes et al., 2003; Prażmo et al., 2003). Additionally, the transfer of bacteria from wastewater to the air has been shown to occur throughout the treatment process with the greatest transfer often occurring during the wastewater aeration step (Filipkowska et al, 2000; Pascual et al., 2003; Fernando and Fedorak, 2005; Sánchez-Monedero et al., 2008; Korzeniewska et al., 2009; Wang et al., 2018). While wastewater bioaerosols are considered a possible route of worker exposure to biohazards, little work has been done to understand the contribution of bioaerosols to the transmission and possible exposure to ARGs. Studies have shown that bioaerosols generated

within agricultural settings can aid in the dispersal of ARB/ARGs (Just et al., 2012; McEachran et al., 2015) and one study showed the presence of a sulfonamide resistance gene (*sul2*) in bioaerosols generated during the sludge thickening stage of wastewater treatment (Li et al., 2016). Coupling these lines of evidence with the knowledge that WWTPs can harbor high concentrations of ARB, it is likely that bioaerosols generated during municipal wastewater treatment represent a significant route of disseminating ARGs into surrounding urban environments.

The main goal of this work was to better define the contribution of WWTP bioaerosols to potential environmental distribution of ARGs. Replicate bioaerosol samples were collected immediately upwind and downwind from the aeration tanks of a municipal wastewater treatment plant that uses secondary treatment based on mixed coarse and fine bubble aeration of activated sludge. For source-to-air comparison, replicate liquid sludge samples were obtained from the aeration tanks. For all samples, a combined culture-dependent and culture-independent approach was used to test for the presence and quantity of 84 ARGs conferring resistance to a range of antibiotic classes and to characterize the taxonomic diversity of the microbial community. Results from these studies will further define the contribution of bioaerosols to antibiotic resistance gene distribution and provide a foundation for modeling the fate and transport of antibiotic resistance genes from a WWTP source into the surrounding environment.

Methods

Sample site and meteorological conditions

The WWTP site that was examined in this study is located in the southeast USA along the coast of South Carolina. This WWTP uses secondary treatment with coarse and fine bubble aeration of activated sludge and is capable of treating approximately 15 million liters of municipal waste per day (MLD). This site was chosen because it uses similar treatment technology as many existing WWTPs, the coastal location provides predictable wind patterns, and its small scale will limit model complexity. During sampling, the meteorological conditions were as follows: average temperature: 13°C, average wind speed: 13 km h^{-1} from north-northwest direction, average humidity: 70%, and zero precipitation.

Field sample collection

To examine the dispersal of ARGs from activated sludge tanks, bioaerosol samples were collected immediately downwind from sludge aeration tanks (Figure 1). Bioaerosol samples were also collected upwind from all WWTP processes to provide an estimate of the background prevalence of ARGs. Due to the prevailing wind direction, the control site was located immediately adjacent to the northern boundary of the WWTP, providing a sampling area with minimal influence of any WWTP process. At both upwind and downwind sites, replicate bioaerosol samples were collected on polytetrafluoroethylene (PTFE) filters for culture-independent analysis and on gelatin membrane filters for culture-

dependent analysis. For culture independent analysis of the microbial community taxonomic diversity and ARG prevalence, nine PTFE samples were collected simultaneously at both upwind and downwind locations for 90 mins with a flow rate of 56 L/min resulting in a volume of 5,040 L of air filtered per sample and 45,360 L of total air filtered at each location. Following sampling, PTFE filters were immediately placed in sterile 15 ml conical tubes containing 8ml of Qiagen AL buffer, stored on ice for transport to the laboratory, and subsequently stored at -80˚C until further processing. For culture-dependent microbial community and ARG analysis, six samples were collected at both upwind and downwind locations on gelatin membrane filters using Sartorius MD8 Airscan air samplers at a flow rate of 125 L/min for 30 mins, resulting in 3,750 L of filtered air per sample and 22,500 L of total filtered air at both locations. After sampling, gelatin membrane filters were individually stored in sterile bags and cooled for transport to the laboratory. To characterize the potential source of downwind bioaerosols, six samples of liquid activated sludge were collected directly from the surface of the sludge in sterile 50 ml conical tubes. The sludge was centrifuged for 10 min at 21,000 x g and the pellet resuspended in 8 ml of Qiagen AL buffer and stored at -80˚C for further processing.

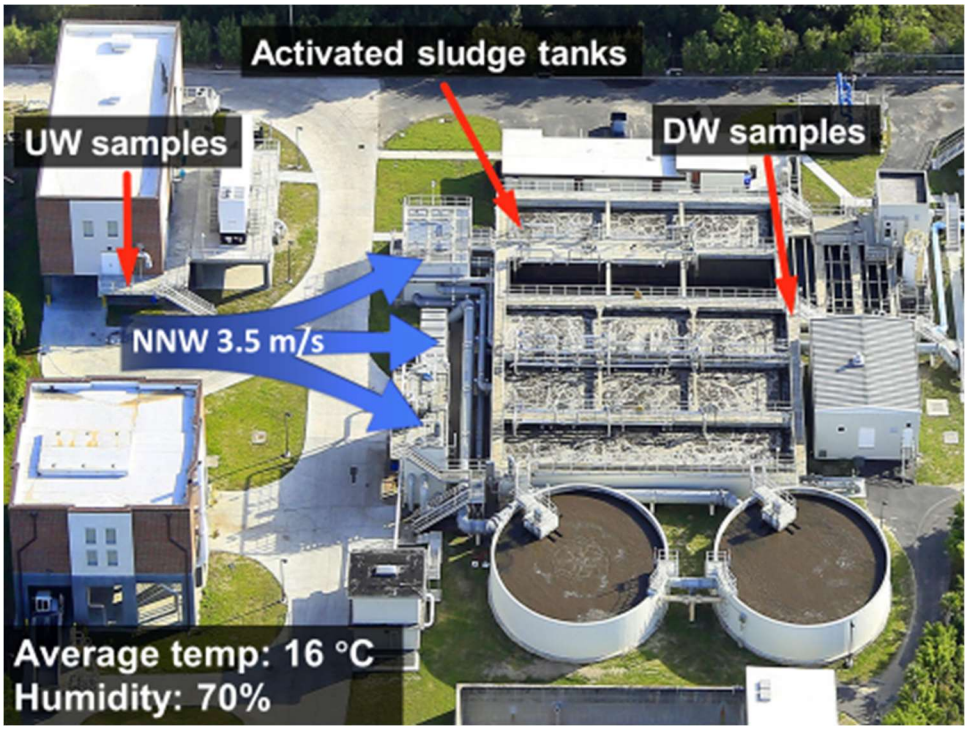


Figure 4.1. Layout of the examined WWTP showing the sampling locations and prevailing wind direction.

Isolation of culturable bacteria

To isolate culturable bacteria from upwind and downwind samples, gelatin filters were incubated on 1% Luria-Bertani (LB) agar plates containing 0.0125 % of cycloheximide and incubated at 28˚C for 72 hours. Following incubation, plates were washed twice with 1% LB and the wash collected in 15 mL centrifuge tubes. Four ml of each plate wash was combined with an equal amount of 50% glycerol and aliquoted into 2 ml freezer vials for long-term storage at -80˚C. The remaining wash was aliquoted into 15 ml tubes and centrifuged for 10 min at 21,000 x g. The cell pellets were resuspended in 8 ml of Qiagen AL buffer and stored at -80˚C for further processing.

Nucleic acid extraction

DNA extraction from PTFE filters, culture-based cell pellets, and liquid sludge pellets was performed using a combination of bead-beating, freeze-thaw, and the Qiagen DNeasy Blood & Tissue DNA extraction kit. Briefly, a 2:1 mixture of 1 mm and 0.1 mm DNase- and RNase-free silicon-carbide beads (Biospec Products, Bartlesville, OK) was added to each sample followed by bead-beating for 10 min. Following bead-beating, samples were exposed to three freeze-thaw cycles with freezing in liquid nitrogen for 1 min and thawing at 70˚C for 5 minutes. Samples were then centrifuged for 10 min at 8,000 x g and supernatant passed through the DNeasy mini spin column and DNA eluted with AE buffer following the manufacturer's instructions. DNA quality and quantity was measured using a NanoDrop spectrophotometer and a Qubit fluorometer (ThermoFisher Scientific). Isolated DNA was then used for qPCR and DNA sequencing to examine the presence and quantity of ARGs and the microbial community composition, respectively.

Microbial community antibiotic resistance gene identification

The identification of ARGs in upwind and downwind bioaerosols, liquid sludge, and cultured cells was examined using the Antibiotic Resistance Genes Microbial DNA qPCR arrays (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. These arrays provide high-throughput profiling of 84 genes that represent major classes of ARGs (i.e., aminoglycoside, beta-lactam, fluoroquinolone, macrolide-lincosamide-streptogramin B, tetracycline, vancomycin, and multidrug resistance classifications). Each 25 µl qPCR reaction

consisted of 12.5 µl of Microbial qPCR Mastermix (Qiagen) containing the HotStart DNA Polymerase, 5 ng of template DNA, and microbial DNA-free water. Reaction mixtures were added into each well of the 96-well ARG array plate containing primer-probe mixtures specific for each tested gene. Pan-bacteria and PCR control wells provided positive controls to test for the presence of bacterial DNA and the absence of any PCR inhibitors. Thermal cycling was performed on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) with the following conditions: Initial PCR activation at 95°C for 10 mins followed by 40 cycles consisting of denaturation for 15 s at 95°C and annealing and extension for 2 min at 60°C. A threshold cycle (C_T) value of 37 was used and baselines were manually set for cycles 8-20 with a threshold fluorescence setting of 0.2. Dissociation curves were generated following reactions and qPCR amplicons were analyzed using gel electrophoresis to examine the specificity of each reaction. The ARGs within each sample were identified using the DC_T method against a no-template control sample as outlined in the Excel Data Analysis Software (Qiagen). The Jaccard index of similarity based on the presence or absence of individual ARGs was used to examine the similarity in ARG profiles observed for each sample type.

Microbial community antibiotic resistance gene quantitation

Microbial DNA qPCR assays (QIAGEN) were used for quantitative comparison of each ARG identified in the downwind samples as compared to upwind and activated sludge samples. Each 25 µl qPCR reaction consisted of 12.5 µl of Microbial qPCR Mastermix (Qiagen) containing the HotStart DNA

Polymerase, 1 µl of Microbial DNA qPCR Assay (contains primer-probe sets unique for each tested ARG), 5 ng of template DNA, and microbial DNA-free water. Thermal cycling was performed on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) using the conditions and settings described in the previous section with dissociation curves and gel electrophoresis used to assess reaction specificity. For quantitation of ARG copy number a six-point C_T standard curve for each gene was generated using triplicate wells of a positive control containing 0, 500, 1000, 2000, 4000, 8000 copies of the tested gene. The C_T values for replicate upwind, downwind, and activated sludge samples were then compared against the standard curve to determine the number of ARG copies for each sample. To minimize potential bias while comparing across different sample types (i.e., liquid and air), ARG copy numbers were normalized to ng of total DNA for each sample. ARG copy numbers were then log transformed and data visualized as grouped box plots generated using the 'ggplot2' package (Wickham, 2016) of the R software (R Core Team, 2018). Analysis of variance (ANOVA) and Tukey's HST were performed using R software to analyze the effects of sample location (UW, AS, DW) on the abundance of each ARG and statistically significant differences were accepted at $p < 0.05$.

Microbial community 16S rRNA gene profiling

From the extracted nucleic acids, the V4 hypervariable region of the 16S rRNA gene was amplified from each sample using the 515F-Y forward primer (5'-GTGYCAGCMGCCGCGGTAA) and the 926 reverse primer (5'-

CCGYCAATTYMTTTRAGTTT) (Parada et al, 2015). Unique multiplex identifier (MIDs) tags were added to the reverse primer sequence to allow multiplexing of samples during Illumina Miseq runs. Triplicate 50 µl NEBNext® High-Fidelity 2X PCR Master Mix reaction mixtures contained 1 ng of DNA, 0.2 µM forward primer and 0.2 µM indexed reverse primer. Cycling conditions for all reactions included a 30 second incubation step at 98˚C, followed by 25 cycles of 98˚C for 10 seconds, with an annealing temperature of 55˚C for 25 seconds and extension at 72˚C for 10 seconds, and a final extension at 72˚C for 5 minutes. Amplicons were purified using the QIAquick PCR purification kit (Qiagen) and quantified with a Qubit 2.0 fluorometer (Life technologies, Grand Island, NY, USA). Indexed amplicons from all samples were combined on a 1:1 concentration ratio. The combined sample was analyzed using a Qubit fluorometer to measure concentration and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) to measure amplicon size distribution. The final library was diluted to 17 pM and analyzed on an Illumina MiSeq DNA sequencer (Illumina, Inc., San Diego, CA, USA) using the MiSeq Reagent Kit v3 providing 2 x 300 bp paired end reads. The resulting amplicon sequences were analyzed using mothur [v.1.33.0; (Schloss et al., 2009)] following a modified version of the MiSeq SOP (Kozich et al., 2013). After paired-end reads from all libraries were assembled, sequences not matching quality criteria (maximum ambiguities = 0, £ 8 homopolymers, ambiguous length ³ 300 or <700bp) were culled using the screen.seqs command. Non-chimeric sequences were dereplicated and aligned using Silva Archaea and Bacteria databases trimmed to

the V4 region. Sequences were assigned to operational taxonomic units (OTUs) with a sequence similarity threshold of 97% identity using the cluster.split command and subsequently classified. Heatmaps were used to visualize the relative abundance of classified sequences across samples and constructed using R software (R Core Team, 2018) with the heatmap2 command in gplot (Warnes et al., 2012). The Shannon and inverse Simpson indices of diversity, the Bray-Curtis dissimilarity coefficient (BC), and the shared Chao richness indicator were calculated at the 0.03 level using Mothur (v.1.33.0). The Bray-Curtis index and percent similarity of the microbial community profiles for the different samples was calculated by (1-BC)*100. The Kruskal-Wallis rank sum test was used to assess differences in the alpha diversity of samples based on location and pairwise comparisons were made using the Wilcoxon rank sum test with FDR p-value correction. Permutational multivariate analysis of variance (PERMANOVA) was applied to Bray-Curtis distance matrices using R with the Vegan package (Oksanen et al., 2017) to test the differences between community diversity and sampling location.

Antibiotic resistance gene dispersion

The dispersion pattern of ARGs emitted from the WWTP was determined using an approach of coupling the wind rose at the site with the sampling data. The inputs to the model was meteorology data: the wind velocity and direction and emission rate of ARGs from the WWTP. The wind velocity and direction were based on the wind rose available for the location closest (70 m) to the

sampling site. ARG emission rate was calculated using total non-transformed ARG copy numbers generated using qPCR quantitative assays of the DW bioaerosols per hour of sampling. ARGs that were identified in both UW and DW samples were not included in the emission rate as the source of these ARGs was inconclusive. Based on this approach, the emission rate was estimated to be ~10,620 total ARGs per hour. For the mathematical approach, the following assumptions were applied: 1) no deposition, 2) the terrain had no impact, 3) continuous emission over the time period under consideration and 4) the ARGs were conveyed by aerosols that acted as tracers (i.e. the flow dynamics controlled the dispersion pattern). The quantity or total abundance of the ARGs (GC_N) and the distance traveled (*D, km*) by the genes was calculated using the equation: where *ER* = emission rate (total ARG abundance per hour), *t* = time (h), and *WS* = wind speed (km h^{-1}). The calculations were done using Microsoft Excel and the openair (Carslaw and Ropkins, 2012) and plotrix (Lemon, 2006) packages of R.

Results

Identification of antibiotic resistance genes in WWTP sludge and bioaerosols

A qPCR based array system was used to examine the presence of ARGs in WWTP activated sludge (AS) and bioaerosols collected upwind (UW) and downwind (DW) from the activated sludge aeration tanks. Out of the 84 tested genes, AS showed the greatest frequency of ARG detection (50%; 42

ARGs) followed by the DW samples (20%; 17 ARGs) and the UW samples (7%; 6 ARGs). The beta-lactam resistance genes (BLA) were the most frequently detected ARGs, comprising 60% of genes identified in AS samples, 54% in DW, and 34% in UW. Further analysis indicated that among the BLA genes, those encoding serine-utilizing hydrolases (Ambler classes A, C, and D) were the most prevalent with Ambler class B genes encoding metallo-utilizing hydrolases detected less frequently. Other frequently detected ARGs were those conferring resistance to fluoroquinolones (Fluoro), aminoglycosides (Aminogly), tetracyclines (Tetracyc), and macrolide/lincosamide/streptogramin (MLS).

At the individual gene level, the Jaccard index of similarity showed that the activated sludge AR profile is most similar to that found in the DW samples (68% similar) as compared to UW samples (AS vs UW 13% similar) (Figure 3.2). The occurrence of ARGs across samples is listed below and separated by beta-lactamase class or the antibiotic classes within which individual genes confer resistance.

Beta-lactamases: Activated sludge samples showed the presence of a wide range of genes across beta-lactamase classes A and D encoding extended spectrum beta-lactamases (ESBLs) that have activity against late generation cephalosporin antibiotics. Among these genes, the plasmid mediated *GES, TLA-1, VEB, OXA-10, OXA-2* ESBL-encoding genes were found in both AS and DW samples but not UW samples. Among these classes, one AR gene (*OXA-60*) was found in UW, DW, and AS samples. Among the class C (AmpC) beta-lactamases, six plasmid-encoded genes that are often associated

with *Enterobacteriaceae* and confer resistance to broad and extended spectrum cephalosporins were observed in the AS. Additionally, genes encoding the FOX-type enzymes (*FOX*) that are especially active against cefoxitin and the constitutively expressed *MIR* genes were also observed in the DW air samples. No class C beta-lactamase genes were observed in the UW samples. Three genes encoding class B metallo-beta-lactamases were observed among all samples. The *ccrA* gene (also called *cfiA*) that is commonly associated with carbapenem resistance in *Bacteroides fragilis* was only observed in AS samples. The divergent integron-encoded *IMP-12* gene conferring resistance to high levels of imipenem was observed in both AS and DW samples while the *IMP-5* gene was only observed in the UW air samples.

MLS: Among the macrolide, lincosamide, and streptogramin B (MLS) resistance genes, the rRNA methylase encoding *ermB* and *ermC* genes were observed in AS, DW, and UW samples while genes encoding efflux pumps (*mefA* and *msrA*) were only observed in AS and DW samples.

Tetracyclines: Genes encoding efflux pumps that confer resistance to tetracyclines (*tetA* and *tetB*) were also only observed in AS and DW samples.

Fluoroquinolones: Seven genes conferring resistance to fluoroquinolones (e.g., ciprofloxacin) were identified in the sludge and air samples. Four groups of the plasmid-mediated *qnr* genes (*qnrA, qnrB, qnrD,* and *qnrS*) encoding pentapeptide repeat proteins were observed in the AS samples with the *qnrS* gene also identified in DW samples and the *qnrB-1* group identified in AS, DW, and UW samples. Additionally, the plasmid-mediated *aac(6')-Ib-*

cr gene encoding a variant of the aminoglycoside acetyltransferase was also identified in the AS and DW samples.

Aminoglycosides: The potential for aminoglycoside enzymatic inactivation by acetyltransferases (*aacC1* and *aaC2*) was observed only in AS samples while genes encoding nucleotidyltransferases (*aadA1*) were identified in AS, DW, and UW samples.

Vancomycin: Potential resistance to vancomycin was identified only in the AS samples by the presence of the *vanB* gene encoding a D-Ala-D-Ala ligase homolog that can synthesize D-Ala-D-Lac as an alternative substrate for peptidoglycan synthesis.

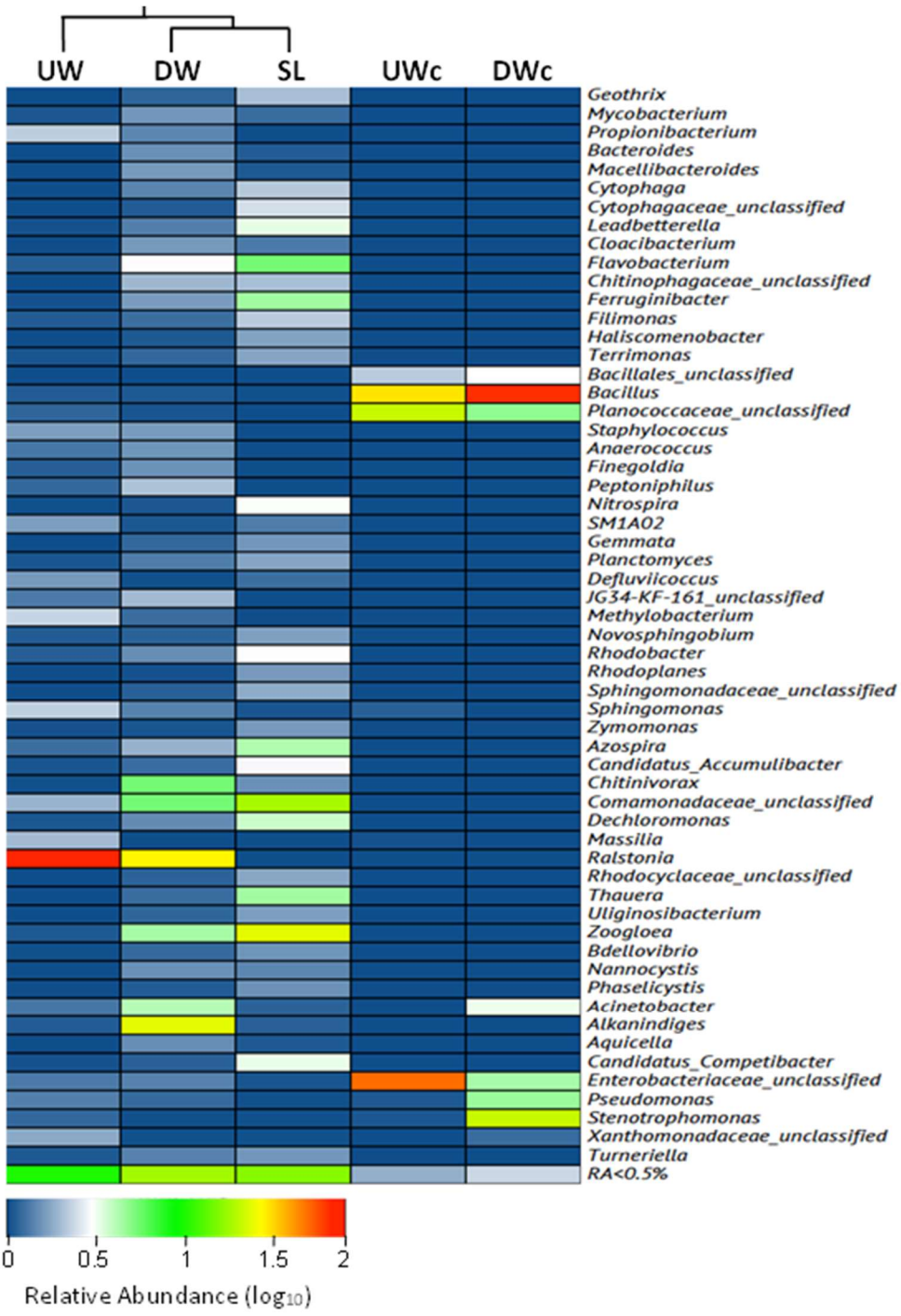


Figure 4.2. Heatmap of Relative abundance of bacterial DNA present in Upwind air samples (UW), Downwind air samples (DW), activated sludge (SL), Upwind cultured air samples (UWc), and Downwind cultured air samples (DWc).

Class A-BLA	UW	DW	AS	DWc	UWc
BES-1			●		
CTX-M-1 Group			●		
GES		●	●		
KPC			●		
SHV(156G)			●		
SHV(238G240E)			●		
SHV(238S240E)			●		
SHV(238S240K)			●		
TLA-1		●	●		
VEB		●	●		

Macrolide^{R} Lincosamide^{R} Streptogramin_b^{R}	UW	DW	AS	DWc	UWc
ermB	●	●	●	●	
ermC	●	●			
mefA	●	●	●	●	
msrA			●	●	

Tetracycline^{R}	UW	DW	AS	DWc	UWc
tetA	●	●	●		
tetB			●		

Class B-BLA	UW	DW	AS	DWc	UWc
ccrA			●		
IMP-12 group		●	●		
IMP-5 group	●				

Fluoroquinolone^{R}	UW	DW	AS	DWc	UWc
AAC(6)-Ib-cr		●	●		
QnrA			●		
QnrB-1 group			●		
QnrB-5 group		●	●		
QnrB-8 group			●		
QnrD			●		
QnrS		●	●		

Class C-BLA	UW	DW	AS	DWc	UWc
ACT-1 group			●		
CMY-10 Group			●		
FOX		●	●		
LAT			●		
MIR		●	●		
MOX			●		

Aminoglycoside^{R}	UW	DW	AS	DWc	UWc
aacC1			●		
aacC2			●		
aadA1	●	●	●	●	

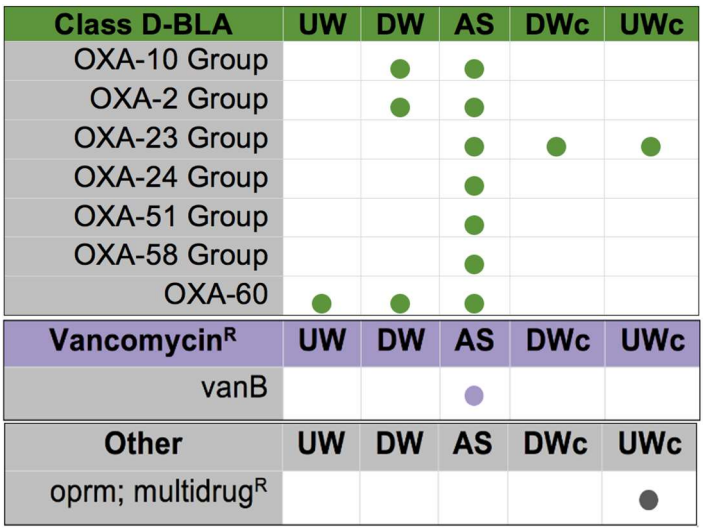


Figure 4.3. Identification of specific antibiotic resistant genes in WWTP upwind (UW), downwind (DW), activated sludge (AS), cells cultured from UW (UWc) and DW (DWc).

Quantitation of antibiotic resistance genes in WWTP sludge and bioaerosols

To better define the potential influence of sludge aeration on the antibiotic resistance profile of WWTP bioaerosols, the number of copies of each ARG identified in the DW air samples was compared to the number of copies found in liquid sludge samples and the UW control air samples . Analysis of variance indicated that the abundance of ARGs differed significantly ($p < 0.05$) based on sample location. Among the Class A BLA genes, the *GES*, *TLA-1*, and *VEB* genes were not identified in UW control samples but were present in DW (1.3, 2.1, and 3.2 copies, respectively) and in AS samples (1.7, 1.2, and 2.6 copies, respectively). While there was no significant difference (Tukey's HST $p > 0.05$) in the abundance of the GES gene between the AS and DW samples, both the *TLA-1* and *VEB* genes were significantly higher ($p < 0.05$) in abundance in the DW samples. A similar trend was observed for the

class B *IMP-12* gene with DW samples containing higher copy numbers (3.5 copies) compared to AS samples (1.9 copies). The class C BLA genes, *MIR* and *FOX*, were the lowest abundant ARGs detected, with abundances ranging from 0.2 to 0.6 copies in the AS and DW samples. The most abundant ARG identified was the class D-BLA *OXA-60* gene which had an abundance of approximately 4.0 copies in UW and DW samples as compared to 1.9 copies in the AS samples. The *OXA10* and *OXA2* genes were higher in abundance in AS samples (3.0 and 2.7 copies, respectively) as compared to DW samples (2.5 and 1.3 copies, respectively). Among the MLS ARGs, the *mefA* gene was not present in UW samples but had similar abundances of approximately 2.3 copies in AS and DW samples. The *ermB* abundance was significantly different ($p < 0.05$) between the UW (1.1 copies) and DW (2.3 copies) air samples but both were not significantly different from the AS samples (1.7 copies). However, the *ermC* gene was significantly higher ($p < 0.05$) in both UW (2.8 copies) and DW (2.4 copies) air samples as compared to AS samples (0.4 copies). The tetracycline resistance gene (*tetA*) was most abundant in the AS samples (3.6 copies DNA) as compared to the DW samples (2.3 copies). Among the fluoroquinolone resistance genes, the *AAC(6)-Ib-cr* gene had similar abundances within AS and DW samples (2.3 and 2.6 copies, respectively) while the *QnrS* gene was more abundant in DW samples (3.1 copies) as compared to AS samples (2.5 copies). Additionally, the abundance of the *QnrB-5* gene was not significantly different between the UW (2.5 copies) and DW (1.5 copies) air samples but was significantly lower ($p <$

0.05) in abundance in the AS samples (0.4 copies). The aminoglycoside resistance gene, *aadA1*, was identified in all samples but was more abundant in DW and AS samples (approximately 2.3 copies) as compared to UW air samples (0.8 copies).

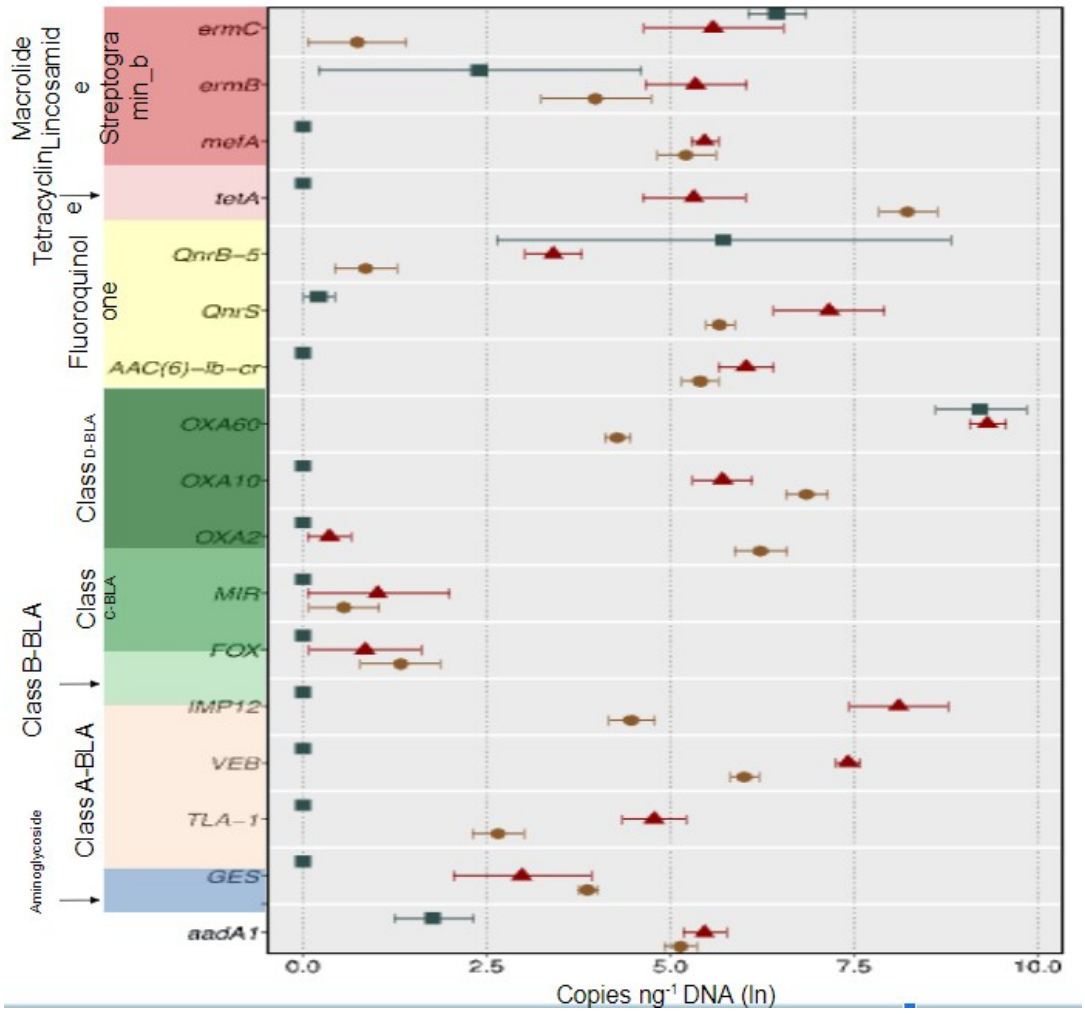


Figure 4.4. The concentration of antibiotic resistant genes identified in Upwind air samples (blue), Downwind air samples (red), and activated sludge (brown)

Microbial community analysis

To examine the possible dispersal of bacteria from liquid sludge into surrounding air, microbial community 16S rRNA gene profiles were generated for UW control air samples, liquid AS, and DW air samples. Based on the Shannon and inverse Simpson indices, the AS community showed the greatest microbial diversity at the phylum level followed by the DW and UW communities. The most abundant phyla across the UW, AS, and DW samples were the Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes, Planctomycetes, Chloroflexi, Verrucomicrobia, Nitrospirae, Acidobacteria, and Chlorobi. At the genus level, samples showed high richness with most genera having a relative abundance less than 1%. However, 16 genera had a relative abundance greater 1% in some samples [listed in brackets] and were contained within the *Betaproteobacteria* (*Zoogloea* [AS, DW], unclassified_Comamonadaceae [AS, DW], *Thauera* [AS], *Azospira* [AS], *Dechloromonas* [AS], *Chitinivorax* [DW], *Candidatus_Accumulibacter* [AS], *and Ralstonia* [UW, DW]), the *Alphaproteobacteria* (*Rhodobacter* [AS]), the *Gammaproteobacteria* (*Alkanindiges* [DW], *Acinetobacter* [DW], and *Candidatus_Competibacter* [AS]), the Bacteroidetes (*Flavobacterium* [AS, DW], *Ferruginibacter* [AS], and *Leadbetterella* [AS], and the Nitrospirae (*Nitrospira* [AS]). Overall, the Shannon and inverse Simpson indices showed that the microbial diversity was significantly different based on sample location (Kruskal-Wallis rank sum test $p < 0.05$). Pairwise comparisons showed that the diversity for the UW, AS, and

DW sites were unique (Wilcoxon rank sum test $p < 0.05$) with the greatest microbial diversity found in the AS and DW samples followed by UW control air samples. Additionally, PERMANOVA test showed that the microbial community structure at each sample location was different ($p < 0.05$) and pairwise comparisons of the Bray Curtis index and shared Chao showed that the AS and DW communities were more similar (28.9% similar) as compared to the AS and UW communities (3.4% similar). The UW and DW communities were also 28.9% similar, suggesting that the DW samples are equally influenced by the UW and AS microbial communities. While direct links between specific bacteria and ARGs could not be determined, since the same DNA was used in 16S rRNA gene and ARG profiling, the overall ARG profile of each sample could be associated with its community composition.

Comparison of culture-dependent and culture-independent bioaerosol analyses

To explore the differences in the results of using culture-dependent versus culture-independent approaches to characterize the bioaerosol samples, culturable microbes were isolated from cellulose filters and the ARG and taxonomic profiles compared with those generated from the culture-independent analysis. The culture-independent analysis detected more ARGs than the culture-dependent analysis with 17 versus 5 ARGs detected in DW samples compared to DW cultures (DWc) and 6 versus 2 ARGs detected in UW samples as compared to UW cultures (UWc). Among the individual ARGs detected in cultured cells, the *oxa-23*, *msrA*, and the *oprm* genes were detected

in bioaerosols using the culture-based approach but not the culture-independent approach while the remaining genes were detected using both approaches (Figure 3.2). When analyzing microbial community alpha diversity, the Shannon and inverse Simpson indices indicate that the diversity of the culture-based samples (UWc and DWc) was significantly lower (Wilcoxon $p < 0.05$) than the culture-independent samples at the phyla and genera levels. The taxonomic profiles of the UWc and DWc show that the most abundant cultured microbes were affiliated with the Firmicutes and Proteobacteria phyla. At the genus level, genera within the Firmicutes (*Bacillus* and *unclassified_Planococcaceae*) and the *Gammaproteobacteria* (*unclassified_Enterobacteriaceae* and *Pseudomonas,* and *Stenotrophomonas*) were detected at lower abundances in bioaerosols using culture-independent methods (UW and DW) as compared to higher abundances found in the cultured samples (UWc and DWc). Overall, the Bray-Curtis and shared Chao indices indicate that the microbial community structure for the UWc and DWc samples were more similar (24%) to each other than to any of the culture-independent samples.

Antibiotic resistance gene dispersion

The potential dispersion pattern of ARGs emitted from the WWTP was modeled through coupling meteorological data (wind pattern) and ARG emission rate. The emission rate of ~10,620 total ARGs per hour was based on the abundance of ARGs found in DW bioaerosols but not UW control samples. Among the ARGs included in the emission rate, three genes (*QnrS*, *IMP12*, and *VEB*) comprised 83% of the estimated emissions while

an additional nine genes comprised the remaining 17% of ARG emissions. The wind rose at the sampling location showed that 60% of the wind was from the north flowing south towards the plant. Winds originated mainly from the north (N), north north west (NNW), north west (NW) and west (W). Maximum wind speeds between 16 to 20 km h^{-1} occurred about 2% of the sampling period. The dominant wind speed was between 8 to 12 km h^{-1} occurring nearly 40% of the duration from the N, 12% from the NNW, 25% from NW and 5% of the duration from the W. Wind speeds in the range of 4 to 8 km h^{-1} occurred only from the N and NNW directions. The lower half of the plot shows the frequency and total ARG abundance per hour for the wind conditions occurring during the period of sampling. The maximum hourly gene count, >3,000 ARGs was possible 7% of the time towards the south (S) while 1,800 to 2,400 ARGs spread towards the south (S) and south south east (SSE) at frequencies of 15 to 45% and 12 to 25%, respectively. Towards the east (E) the frequency was 8% for 1,200 to 1,800 ARGs and less than 5% frequency for the spread of 1,800 to 2,400 ARGs. The possible spread of ARG emissions over a 24h time period based on wind velocity but independent of wind direction was also determined. These dispersion patterns were calculated based on minimum (5 km h^{-1}), average (10 km h^{-1}) and maximum (20 km h^{-1}) wind speeds. At the minimum wind speed, the model suggests that after a 24 h time period, more than 220,000 ARGs can be dispersed within a 10 km radius around the WWTP. At 80 km from the source the number of ARGs is more than 50,000 and decreases based on distance to the near emission rate of 10,000 ARGs at a distance of 120

km. Simulated increases in wind speed show concomitant increases in ARGs at all distances with greater than 100,000 and 200,000 ARGs occurring at 120 km at wind speeds of 10 and 20 km h^{-1}, respectively.

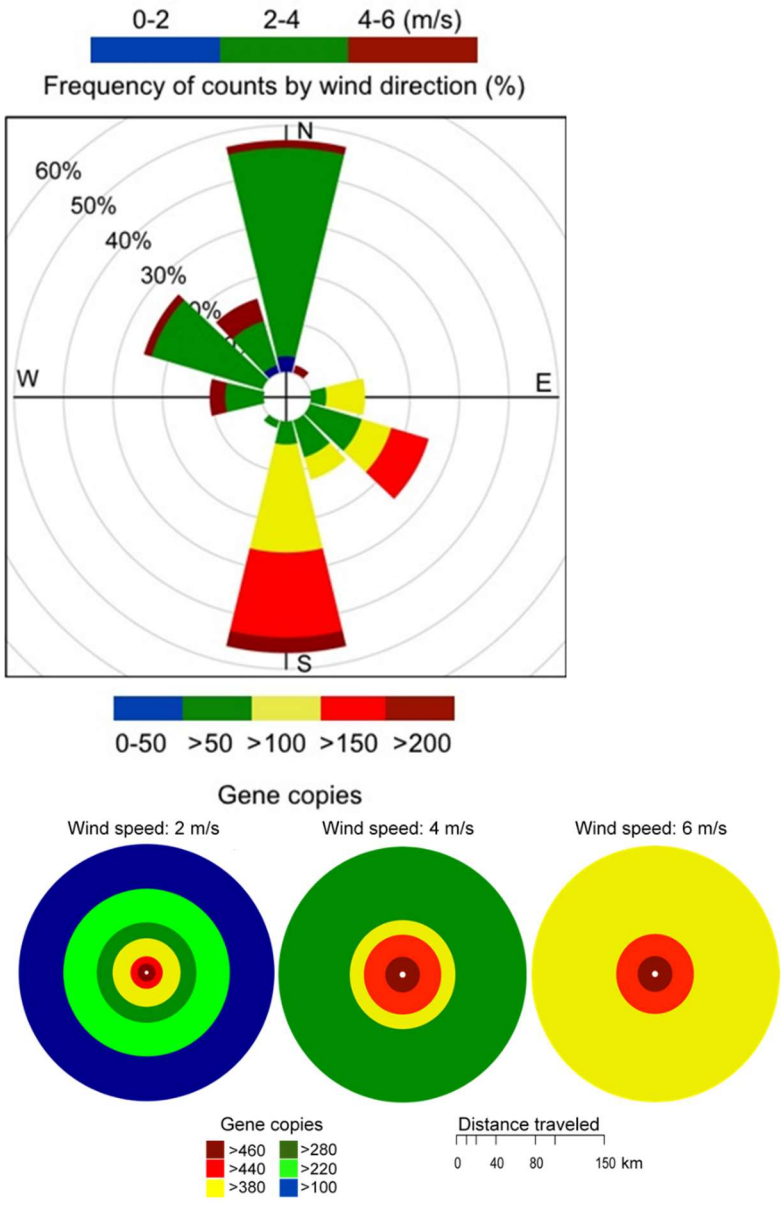


Figure 4.5. Wind rose and dispersion model.

Conclusions

With global antibiotic consumption increasing (Van Boeckel et al., 2014) and resistant infections becoming more prevalent, not only are more extensive antibiotic stewardship programs needed but also better knowledge and surveillance of areas of possible human exposure to ARB and ARGs. The primary finding of our study was that bioaerosols generated during the treatment of municipal sewage can be a potential source for the emission and dispersal of large numbers of a diverse range of clinically relevant ARB/ARGs. While studies are needed to further define the influence of meteorological, terrain, and process variability, our modeling showed that this dispersal may generate an area of localized high occupational human ARB/ARG exposure as well as more widespread deposition into surrounding communities and ecosystems. Lastly, the methods used during sewage treatment at the WWTP investigated in this study are routinely used at other sites, suggesting that similar patterns of bioaerosol ARB/ARG dispersal may be occurring at WWTPs across the developed and developing world and contributing to the trend of increasing global rates of antibiotic resistant infections.

CHAPTER 5:

LONGITUDINAL ANALYSIS OF ANTIBIOTIC USE AND DESCRIPTION OF TWENTY-ONE CLASSES OF ANTIBIOTICS IN SOUTH CAROLINA BETWEEN 2010 AND 2017

Statistical significance of antibiotic use and quantities

The role of antibiotics in medicine in the last 75 years has been decisive in the developments of drugs used against communicable disease, surgical procedures and advances in immunology (Rodríguez-Rojas, Rodríguez-Beltrán, Couce, & Blázquez, 2013). The morbidity and mortality from infectious disease decreased considerably with the inception of penicillin (Ling et al., 2015). The dawn of antibiotics contributed in developing a mentality of conformism and familiarity within clinical practice in the treatment of infectious conditions (Andersson & Hughes, 2012) (Gould, 1999).

An unintended consequence of the broad use of antibiotics was the rapid subsequent antibiotic resistance acquired by bacteria (Spicknall, Foxman, Marrs, & Eisenberg, 2013). Bacteria resistant to penicillin were isolated almost immediately after the first successful treatments with the antibiotic (Berendonk et al., 2015) (Grundmann et al., 2011). Resistance to antibiotics is a capability that bacteria have developed over millions of years of evolution (Martinez, Coque, &

Baquero, 2015)(von Wintersdorff et al., 2016). It can be compared to a system of checks and balances that occurs naturally between microorganisms to maintain equilibrium in the environment (Cheng et al., 2016)(Summers, 2002)(Andersson & Hughes, 2012).

Within the classes of antibiotics, there are broad spectrum or first line antibiotics and targeted antibiotics. Most antibiotics are prescribed for specific health conditions, although there are some that are more widely used. The development of new antibiotics has decreased in the last decades because pharmaceutical companies have noticed a low return on investment. Not long after a new antibiotic hits the market, clinicians start identifying antibiotic resistance to that antibiotic. As a result, antibiotics have started being used based on temporal trends (Raban, et al., 2021). The loss of efficacy of broad-spectrum antibiotics has led to the use of more expensive and targeted antibiotics. The loss of efficacy also represents an increase in morbidity and mortality in developing countries and possibly rural areas. One important factor is the volume of consumption of antibiotics, independent of whether it is being used correctly (Van Boeckel, 2014).

Antibiotic consumption increased 35% between 2000 and 2010, mainly in developing countries, although there was an increased consumption mostly of glycopeptides, carbapenems, polymyxins, and monobactams in many countries irrespective of their income. By contrast, increased cephalosporin and fluoroquinolone consumption was observed mainly in middle-income countries such as India and China. The changing patterns correspond with changes in the

global epidemiology of antibiotic resistance and to the increasing occurrence of some infections that are endemic in developing countries. These trends are suggesting that antibiotic consumption is affected by climate variations, geographical regions, and socio-economic status (Van Boeckel, 2014).

The longitudinal analysis in this study looks at the possible association between antibiotic prescription sales and Years of Potential Life Lost (YPLL), which is a commonly used measure of overall health and is frequently collected at the county level. It also provides information on statistically significant differences within each South Carolina county and between counties, throughout the years in the analysis (2010-2017), which are the years where data is available. The model is used to test any variations throughout time and between the repeated measures. In vague terms, it can be described as running a logistic regression for each of the available measures for the group being studied. The null hypothesis to initially be tested is if there is a statistically significant trend in antibiotic prescription sales from 2010 to 2017 in the counties in South Carolina.

Methods

The data considered in the model is data from the CDDEP data described in the methods section in Chapter 3. In addition, publicly available data from the University of Wisconsin's County Health Rankings project was included. A commonly used indicator for the overall health of a population is the YPLL. (County Health Rankings, 2022). This measure considers premature death as any death before the age of 65. It calculates the years a person could have lived and would have had productive years if it had not been because of their

premature death. In an effort to estimate the role of antibiotics use and sales in a population, for the purpose of this study, YPLL is included as an independent variable in the longitudinal model describing the trend of antibiotic prescription sales from 2010 to 2017.

The null hypothesis describes that there are no significant differences in the number of antibiotic prescriptions sold between the 46 counties in South Carolina from 2010 to 2017 and was tested using a longitudinal analysis with repeated measures and a fixed effect Using R (R Core Team, 2018) with the nlme package (Pinheiro, 2022). The variables included in this model were 'antibiotic prescription sales' as the outcome variable, and 'YPLL', 'quantity of antibiotics', and 'population' as independent variables. Year was the variable used as time for the repeated measures, and included data for the 46 counties in South Carolina from 2010 to 2017.

Missing data methods

After cleaning the data from CDDEP and the publicly available data from County Health Rankings, it was clear that the data provided for YPLL for years 2013 and 2014 was exactly the same. Such coincidence is not normal when working with these types of data, so it was better to replace the data completely for one of the years. To avoid losing one year in this situation, more specifically one year from the middle of the series, using the mean as a replacement for one of the years was plausible. The YPLL data has a normal distribution and using the mean was not going to create any outliers, but was going to reinforce whether the repeated measures had any trends. The method of replacing a

missing value with the mean is called imputation. In the case of this analysis using imputation would bring more benefits than limitations and it is a more favored method than completely eliminating one variable for one whole year from the model (Jakobsen, 2017).

Results

Longitudinal Analysis

The first step in developing the model is testing the null hypothesis with what is called the Unconditional Means Model. This model tests if there is any statistical significance or if the relationship between the data through the years within the counties is different from 0. The only variable included in this model is the outcome variable, without any other factors or a time series.

R Code Model 1

```
mod1 <-
lme(Prescriptions~1,random=~1|Name,data=SC_Counties_pop_year_sum,method="ML")
summary (mod1)
intervals(mod1)
plot (YPLL, Prescriptions)
```

R Output Model 1

```
Linear mixed-effects model fit by maximum likelihood
 Data: SC_Counties_pop_year_sum
       AIC      BIC    logLik
  8212.143 8223.868 -4103.072

Random effects:
 Formula: ~1 | Name
        (Intercept) Residual
StdDev:    133846.2 10784.61

Fixed effects: Prescriptions ~ 1
               Value Std.Error  DF  t-value p-value
(Intercept) 95681.75  19769.43 322 4.839884       0

Standardized Within-Group Residuals:
       Min         Q1        Med         Q3        Max
-7.1401424 -0.2485878 -0.0298026  0.1828655  5.2043247

Number of Observations: 368
Number of Groups: 46
```

From this model, it can be determined that the null hypothesis is rejected: the p-value of 0 means that the mean is statistically significant and different from 0. The scatterplot does show a possible linear association between the variables with a line that has a negative slope. The intraclass correlation coefficient (ICC) value does indicate that clustering is occurring. A high ICC value is an indicator that a repeated measures model (or multilevel model) is appropriate.

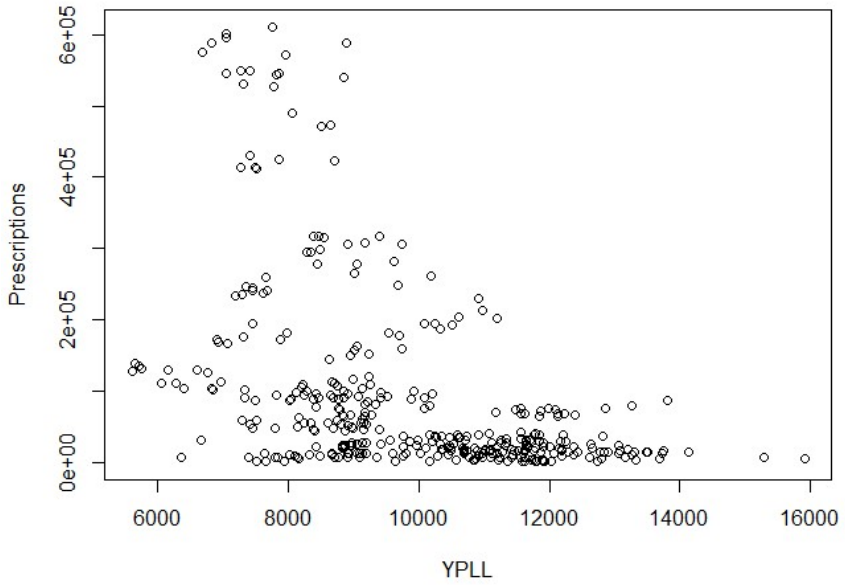


Figure 5.1. Scatterplot of Antibiotic Prescriptions sold and YPLL.

The results from Model 1 indicate that is necessary to determine if the model could use a Model 2 - fixed effect (different slope for each subject) or Model 3 - random effect (the same slope from all subjects). These models are also known as the Unconditional Growth Model. An XY plot of each county is useful to see a graphical representation of the model and determine its accuracy.

R Code Models 2 and 3

```
#Model with a fixed slope
mod2 <- lme (Prescriptions~year, random=~1|Name, data=SC_Counties_pop_year_sum,
method="ML")
summary (mod2)
intervals (mod2)
#Model with a random slope
mod3 <- lme (Prescriptions~year, random=~1|Name, data=SC_Counties_pop_year_sum,
method="ML")
summary (mod3)
intervals (mod3)
xyplot (YPLL ~ year | Name, data = SC_Counties_pop_year_sum, type = c("p","r"))
```

R Output Model 2

```
Linear mixed-effects model fit by maximum likelihood
 Data: SC_Counties_pop_year_sum
       AIC      BIC   logLik
  8213.461 8229.093 -4102.73

Random effects:
 Formula: ~1 | Name
        (Intercept) Residual
StdDev:    133846.3 10773.19

Fixed effects: Prescriptions ~ year
                 Value Std.Error  DF    t-value p-value
(Intercept) -312290.66  495248.2 321 -0.6305740  0.5288
year            202.62     245.8 321  0.8244325  0.4103
 Correlation:
     (Intr)
year -0.999

Standardized Within-Group Residuals:
        Min          Q1         Med          Q3         Max
-7.08196113 -0.22891994 -0.03875583  0.18208195  5.23800196

Number of Observations: 368
Number of Groups: 46
```

R Output Model 3

```
Linear mixed-effects model fit by maximum likelihood
 Data: SC_Counties_pop_year_sum
       AIC      BIC    logLik
  8215.309 8238.758 -4101.655

Random effects:
 Formula: ~year | Name
 Structure: General positive-definite, Log-Cholesky parametrization
            StdDev       Corr
(Intercept) 7.711591e-02 (Intr)
year        6.647583e+01 0.994
Residual    1.073722e+04

Fixed effects: Prescriptions ~ year
                 Value Std.Error  DF    t-value p-value
(Intercept) -312290.66  493200.8 321 -0.6331917  0.5271
year            202.62     245.1 321  0.8265288  0.4091
 Correlation:
     (Intr)
year -0.999

Standardized Within-Group Residuals:
       Min         Q1        Med         Q3        Max
-7.0346073 -0.2344969 -0.0374417  0.1841768  5.2804448

Number of Observations: 368
Number of Groups: 46
```

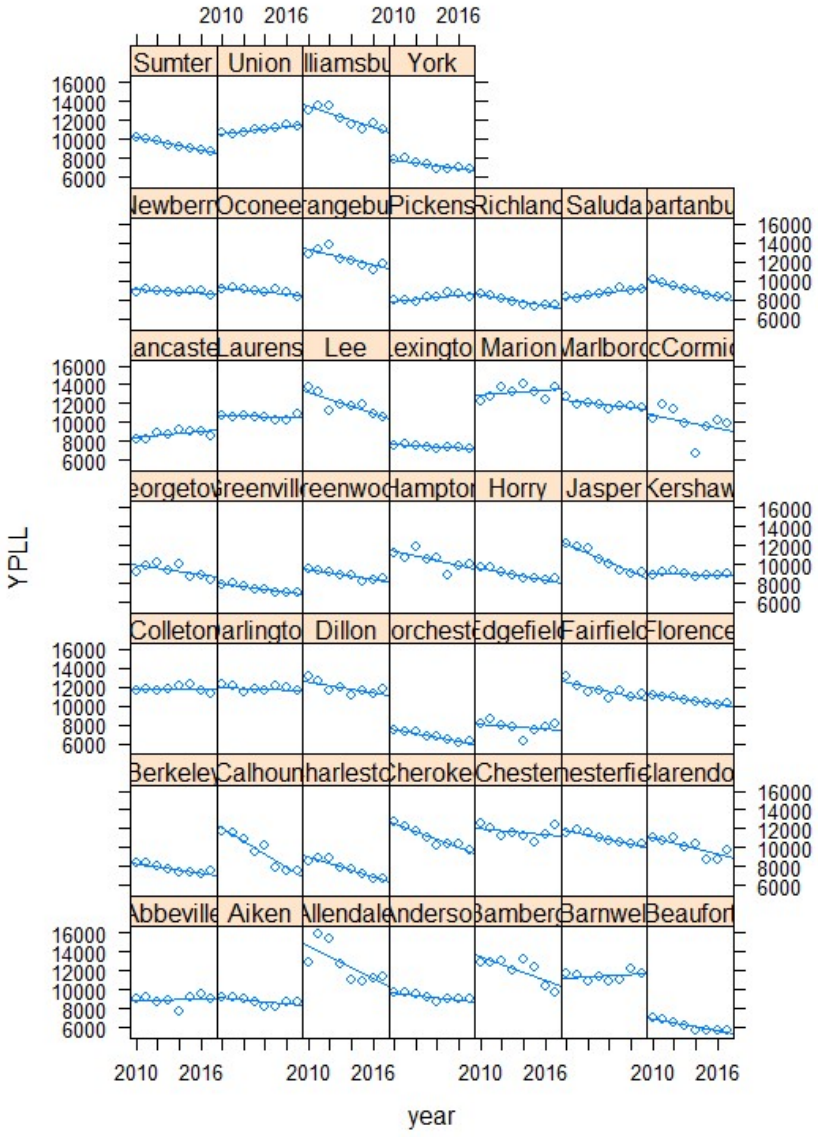


Figure 5.2. XY Plot of YPLL for all the counties (subjects)

The results from the models show that Model 2 is very similar to Model 1 in explaining the relationship of antibiotic use sales though the years. A fixed slope fits the model but it doesn't add a lot of information to the model. From the XY plot we can tell that most of the counties have a slight negative slope. The

results from Model 3 do not improve the results from Model 2. There is a variance-covariance message from the software that does not allow it to calculate the confidence interval. A random slope does not seem to be a good fit for the final model.

The following step in determining the association is to include the independent variables in the model. A repeated measures model also takes into account the interactions between the independent variables. Determining the most accurate model requires running a series of iterations including and excluding different combinations of the variables in the model. The name used for this model is the Conditional Growth Model.

R Code – Model 4

```
mod4 <- lme(Prescriptions~year + Quantity + YPLL + pop + YPLL*pop, random =~1|Name, data
= SC_Counties_pop_year_sum, method = "ML")
summary(mod4)
intervals(mod4)
```

R Output – Model 4

```
Linear mixed-effects model fit by maximum likelihood
 Data: SC_Counties_pop_year_sum
       AIC      BIC    logLik
  7458.941 7490.206 -3721.471

Random effects:
 Formula: ~1 | Name
        (Intercept) Residual
StdDev:    9210.268 4821.162

Fixed effects: Prescriptions ~ year + Quantity + YPLL + pop + YPLL *
pop
                Value Std.Error  DF  t-value p-value
(Intercept) -535563.2 274721.36 317 -1.94948  0.0521
year            269.7    135.71 317  1.98709  0.0478
Quantity          0.0      0.00 317 43.28317  0.0000
YPLL             -0.7      0.41 317 -1.72164  0.0861
pop              -0.1      0.04 317 -2.88927  0.0041
YPLL:pop          0.0      0.00 317  5.14527  0.0000
 Correlation:
         (Intr) year   Quntty YPLL   pop
year     -1.000
Quantity -0.119  0.122
YPLL     -0.335  0.322  0.003
```

```
pop       0.291 -0.300 -0.536  0.356
YPLL:pop -0.285  0.290 -0.159 -0.346 -0.680

Standardized Within-Group Residuals:
        Min           Q1          Med           Q3          Max
-6.571743464 -0.242966155  0.002456094  0.279698944  4.504117655

Number of Observations: 368
Number of Groups: 46

Approximate 95% confidence intervals

 Fixed effects:
                    lower          est.         upper
(Intercept) -1.071646e+06 -5.355632e+05  5.200111e+02
year         4.847318e+00  2.696769e+02  5.345066e+02
Quantity     2.385566e-02  2.498194e-02  2.610822e-02
YPLL        -1.509557e+00 -7.075693e-01  9.441789e-02
pop         -1.776171e-01 -1.060157e-01 -3.441436e-02
YPLL:pop     1.245356e-05  2.006228e-05  2.767101e-05
```

The output from Model 4 shows an improvement in the ability of the model to explain the data from the previous models (Table 5.1). The overall p-value for the mean decreases, almost below the .05 value. Most importantly, three (3) of the covariates were statistically significant (p-value < 0.5) and the interaction between YPLL and population was also statistically significant. The value for the ICC (0.78) means that the model can explain 78% of the relationship between antibiotic prescriptions sold and the covariates included. The model is describing that there is an indirect relationship between antibiotic prescriptions sold and YPLL. On average, when antibiotic sales increase, YPLL or premature death, decreases, and it is adjusted for population numbers (increase or decrease). The model is strengthening the concept that an increase in antibiotics used should represent a decrease in deaths from preventable deaths due to bacterial infections.

The ability to use different classes of antibiotics allows the medical field to prevent and cure these infections. Unfortunately, as it will be discussed in the

next chapter, the curve described in this study is starting to change in many parts of the world. Antibiotics are no longer being effective against common infections and the YPLL is increasing instead of decreasing (WHO, 2019). An important lesson and applicability from this analysis is knowing that increased surveillance is needed and that it is critical not to arrive to the point where the curve or slope between antibiotic prescriptions sold and YPLL starts shifting.

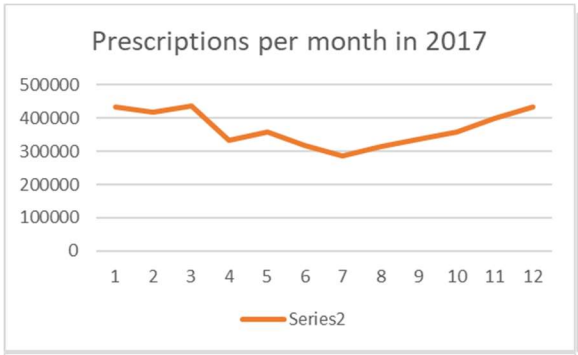
Table 5.1. Comparison of models in the longitudinal analysis

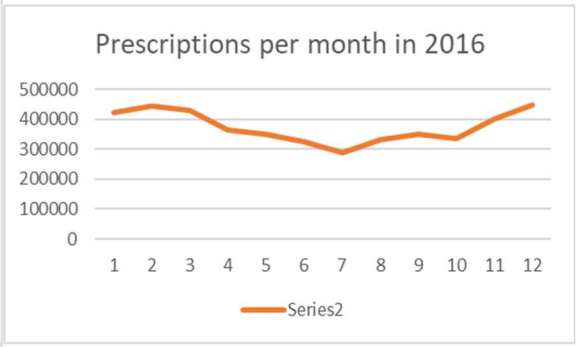
	Model 1	**Model 2**	**Model 3**	**Model 4**
p-value	0	0.413	0.5271	0.0521
logLik	-4103.072	-4102.73	-4101.655	-3721.471
ICC	0.9935496	0.9935632	0.9997765	0.7849261
CI	(9983.035, 11650.556)	(9972.457, 11638.212)	n/a	See output list of CI for covariates

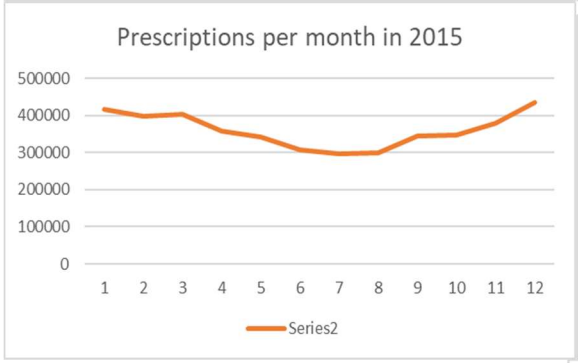
Seasonal Trends

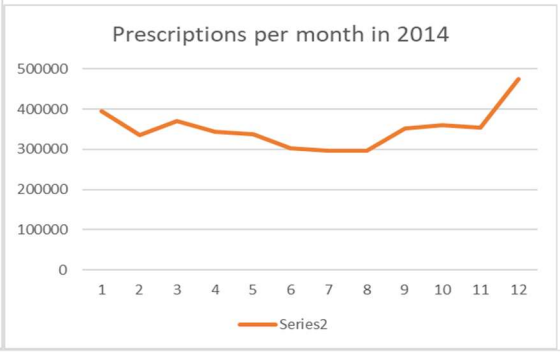
In this portion of the study, data from CDDEP from 2010 to 2017 was also utilized. The plots of the number of antibiotic prescriptions sold per month for every year were created in Excel (2018). Figure 5.3 shows that there is a distinctive seasonal pattern in the sale of antibiotics occurring every year. Sales of antibiotics are higher in the first months of the year, with a downward slope toward the summer months, and then the curve starts increasing until the last month of the year reaching similar number of sales as the beginning part of the year. It is important to highlight that antibiotic sales follow a seasonal pattern characteristic of influenza or other viral respiratory diseases (Polgreen, 2011; Peteranderl, 2016). Rates of infections from bacterial infectious diseases tend to increase during warmer months and seem to be steady in tropical areas. This

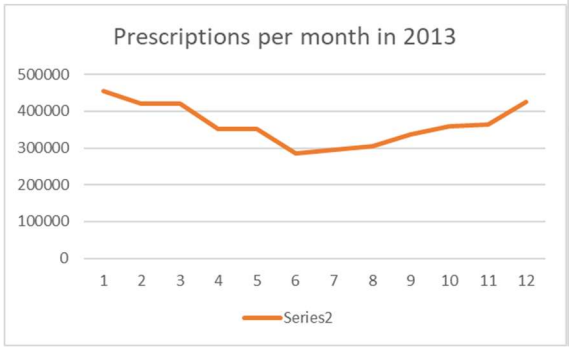
dynamic seen with antibiotic sales is responding possibly to misdiagnosis of disease or over prescription of antibiotics. These data in conjunction with hospital antibiograms can be compared to determine the effectiveness in prescribing antibiotics. It could provide a baseline for the development of antibiotic stewardship programs at the community level.

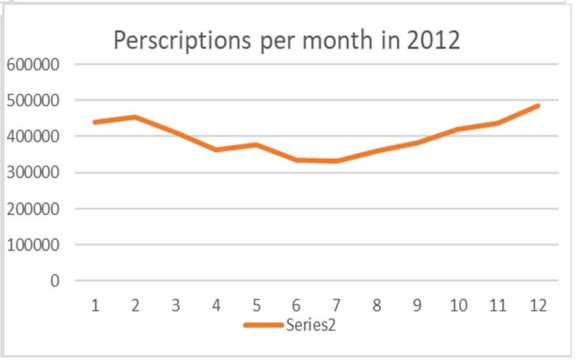












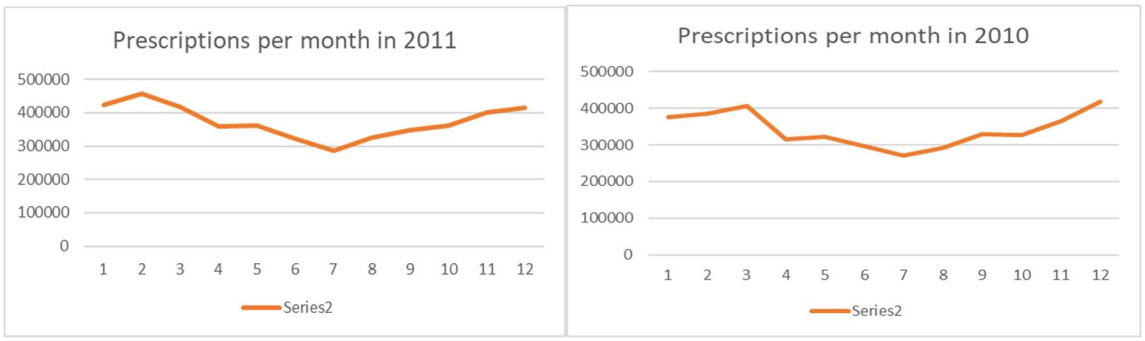


Figure 5.3. Seasonal trends of Antibiotic Sales in SC from 2010-2017.

Description of Antibiotic Classes

The data from CDDEP from 1999 to 2017 is also stratified by antibiotic class. Because not all counties report all classes for every year and do not report all classes in the same manner, it was not possible to include the antibiotic class in the longitudinal analysis. Table 5.2 shows how the classes of antibiotics sold has changed through the years in total percentages. Most of the antibiotics sold include 10 classes (Figure 5.4). Sales of Aminopenicillin, Natural Penicillin, Cephalosporins, and Erythromycin have significantly decreased; sales of Macrolides and Quinolones have increased; and sales of Beta-Lactams, Sulfamethoxazole, and Tetracycline have remained in similar levels from 1999 to 2017. For further research, it could be important to look at the rates and times of appearance of antibiotic resistance found in these classes of antibiotics and compare with the duration it takes for sales to decrease.

The persistence in usage of one antibiotic class can lead to further antibiotic resistance for that antibiotic. The eighteen (18) years of information on antibiotic classes in this data provide significant information to understand when

some antibiotics were more frequently used than others, if there is a possible interaction that could be contributing to antibiotic resistance between the use of certain classes of antibiotics, and which antibiotics could be contributing at a higher level to antibiotic resistance. A limitation of only looking at usage data to identify antibiotic resistance is that there is also a possibility that an antibiotic was discontinued because of the implementation of policy or from a clinical advancement. By including antibiotic classes in the longitudinal analysis, it could be possible to determine if those factors affected equally or deferentially the use of certain antibiotic classes. In order to perform those types of analysis, a standardize way of reporting antibiotic classes is needed.

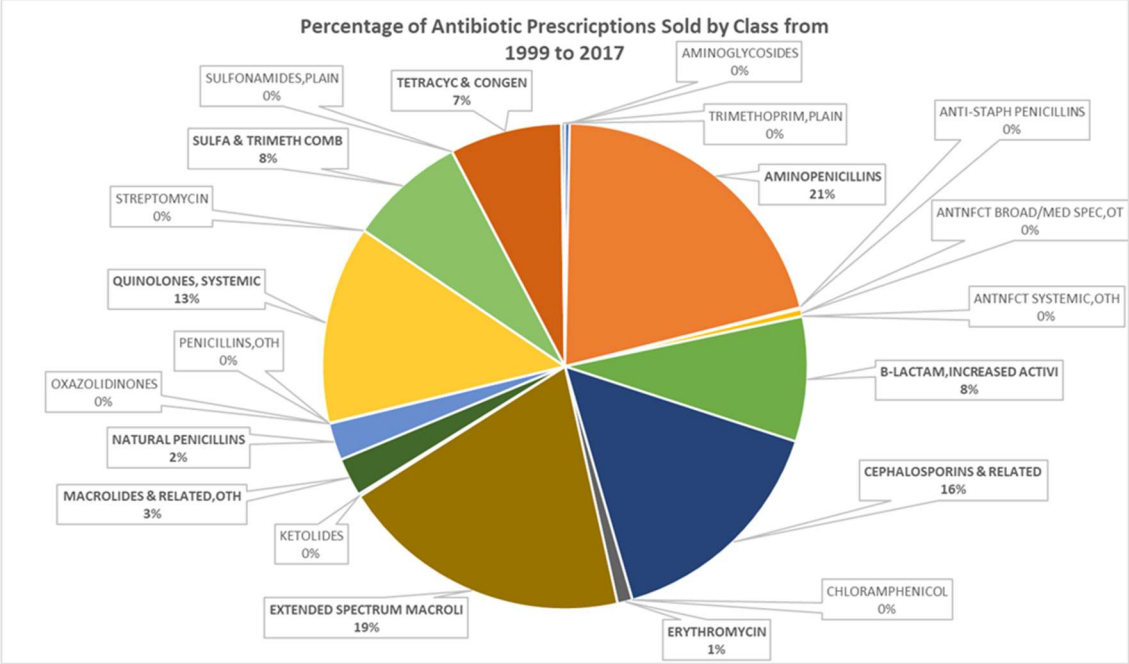


Figure 5.4. Percentage of Antibiotic Class Prescribed from 1999 to 2017.

Table 5.2. List of classes of antibiotics and clinical use during the period of analysis 1999-2017

Class	Clinical Use	Years When Reporting is Missing	Number of Counties that did not Report
AMINOGLYCOSIDES ***Krause, Kevin M et al. "Aminoglycosides: An Overview." Cold Spring Harbor perspectives in medicine vol. 6,6 a027029. 1 Jun. 2016***	Natural or semisynthetic broad-spectrum antibiotics derived from actinomycetes. First line agents for antimicrobial chemotherapy, but replaced in the 1980s with cephalosporins, carbapenems, and fluoroquinolones. Recently re-introduced to use in combination with other classes.		
AMINOPENICILLINS ***LiverTox: Clinical and Research Information on Drug-Induced Liver Injury [Internet]. Bethesda (MD): National Institute of Diabetes and Digestive and Kidney Diseases; 2012-. Penicillins (3rd Generation) [Updated 2020 Oct 20].***	Semisynthetic modifications of natural penicillin that have the advantage of a broader spectrum of activity. The aminopenicillins are widely used for therapy of mild-to-severe urinary, respiratory, gastrointestinal tract, skin, bone and joint infections.		
ANTI-STAPH PENICILLINS **Loubet P, Burdet C, Vindrios W, Grall N, Wolff M, Yazdanpanah Y, Andremont A, Duval X, Lescure FX. Cefazolin versus anti-staphylococcal penicillins for treatment of methicillin-susceptible Staphylococcus aureus bacteraemia: a narrative review. Clin Microbiol Infect. 2018 Feb;24(2):125-132.**	Recommended as first-line agents in methicillin-susceptible Staphylococcus aureus (MSSA) bacteraemia. They have started to be phased out by cefazolin due to concerns about their safety profile.		
ANTI-INFECTIVES BROAD/MED SPECTRUM, OTHER	Class of antibiotics used to treat infections that are broad or mid spectrum and are not included in any other class of antibiotics here listed.		
ANTI-INFECTIVES SYSTEMIC, OTHER	Class of antibiotics used to treat infections that are systemic or are targeted and are not included in any other class of antibiotics here listed.		5

B-LACTAM ***Pandey N, Cascella M. Beta Lactam Antibiotics. [Updated 2021 Sep 30]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2022 Jan-.***	Beta-lactam antibiotics are one of the most commonly prescribed drug classes with numerous clinical indications. Make up 65% of the total antibiotics market. Penicillin, Carbapenems, Monobactams, etc.		
CEPHALOSPORINS ***Bui T, Preuss CV. Cephalosporins. [Updated 2021 Aug 31]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2022 Jan-.***	Cephalosporins are beta-lactam antimicrobials used to manage a wide range of infections from gram-positive and gram-negative bacteria. The five generations of cephalosporins are useful against skin infection, resistant bacteria, meningitis, and other infections.		
CHLORAMPHENICOL ***National Center for Biotechnology Information (2022). PubChem Compound Summary for CID 5959, Chloramphenicol.***	Semisynthetic, broad-spectrum antibiotic introduced into clinical practice in 1948, but which was subsequently shown to cause serious and fatal aplastic anemia and is now used rarely and reserved for severe, life-threatening infections for which other antibiotics are not available.	2008-2017	35
ERYTHROMYCIN ***National Center for Biotechnology Information (2022). PubChem Compound Summary for CID 12560, Erythromycin.***	Is an oral broad-spectrum, macrolide antibiotic that has been in common use since the 1950s. Useful in the treatment of community-acquired respiratory infections. Use has phased out due to the availability of other more effective antibiotics with less side effects.		
EXTENDED SPECTRUM MACROLIDES **Myers, Andrew G., Clark, Roger B. (2021). Discovery of Macrolide Antibiotics Effective against Multi-Drug Resistant Gram-Negative Pathogens. Accounts of Chemical Research 2021 54(7), 1635-1645**	Fully synthetic broad-spectrum antibiotics. Are the most widely prescribed antibiotics in the US. With the increasing development of resistance to current therapies and the lack of safe, oral options to treat Gram-negative infections, extended-spectrum macrolides have the potential to provide valuable treatment options.		

KETOLIDES **Zhanel GG, Walters M, Noreddin A, Vercaigne LM, Wierzbowski A, Embil JM, Gin AS, Douthwaite S, Hoban DJ. The ketolides: a critical review. Drugs. 2002;62(12):1771-804.**	Class of macrolides designed particularly to combat respiratory tract pathogens that have acquired resistance to macrolides. The ketolides are semi-synthetic derivatives of the 14-membered macrolide erythromycin A.	2014-2017	
MACROLIDES & RELATED, OTHER	Macrolides and related that are not included in any other classification here listed.		
NATURAL PENICILLINS **Louis S. Fishman, William L. Hewitt, The Natural Penicillins, Medical Clinics of North America, Volume 54, Issue 5, 1970, Pages 1081-1099**	Antibiotics produced biosynthetically. Benzylpenicillin G and phenoxy-methyl penicillin, 'were' the drugs of choice in treating infections due to susceptible organisms because of their proven effectiveness, low cost, ease of administration, readily manipulated dosage schedules, and relatively low incidence of side effects.		
OXAZOLIDINONES ***Bozdogan B, Appelbaum PC. Oxazolidinones: activity, mode of action, and mechanism of resistance. Int J Antimicrob Agents. 2004 Feb;23(2):113-9.***	Synthetic antibiotics active against a large spectrum of Gram-positive bacteria. Resistance to other protein synthesis inhibitors does not affect oxazolidinone activity. Linezolid was the first oxazolidinone used clinically in 2003.		1
PENICILLINS, OTHER	Penicillins that are not included in any other classification here listed.	2009, 2010, 2012-2017	7

QUINOLONES, SYSTEMIC ***Oliphant CM, Green GM. Quinolones: a comprehensive review. Am Fam Physician. 2002 Feb 1;65(3):455-64.***	Can be classified into four generations. First-generation agents, which are used less often today, have moderate gram-negative activity and minimal systemic distribution. Second-generation have expanded gram-negative activity and atypical pathogen coverage, but limited gram-positive activity. Ciprofloxacin remains the quinolone most active against Pseudomonas aeruginosa. Third-generation quinolones retain expanded gram-negative and atypical intracellular activity but have improved gram-positive coverage. Fourth-generation agents improve gram-positive coverage, maintain gram-negative coverage, and gain anaerobic coverage.		
STREPTOMYCIN	Considered an aminoglycoside, it was reported separately in some cases. Because of decrease susceptibility, it has slowly been phased out and possibly reported within the major group of aminoglycosides. Treatment for TB.	2013-2017	42
SULFAMETHOXAZOLE & TRIMETHOPRIM COMBINED ***Kemnic TR, Coleman M. Trimethoprim Sulfamethoxazole. [Updated 2021 Dec 8]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2022 Jan-***	Antibiotic that is very cost affordable and used for many types of illnesses. When used alone, these drugs only act in a bacteriostatic manner. However, when used in the combination of sulfamethoxazole-trimethoprim, they block two steps in the bacterial biosynthesis of essential nucleic acids and proteins, thus can be bactericidal.		

SULFONAMIDES, PLAIN ***LiverTox: Clinical and Research Information on Drug-Induced Liver Injury [Internet]. Bethesda (MD): National Institute of Diabetes and Digestive and Kidney Diseases; 2012–. Sulfonamides. 2017 Dec 5.***	Represent a large class of antibiotics that have multiple clinical uses. The sulfonamides were the first effective antibiotics to be introduced into clinical medicine and have been in use continuously since the 1930's. bacterial resistance to sulfonamides is now common.		5
TETRACYCLINE & CONGENERS ***Smilack JD. The tetracyclines. Mayo Clin Proc. 1999 Jul;74(7):727-9.***	Among the first of the antibiotics to become available 50 years ago, remain widely used. Tetracyclines have bacteriostatic activity against a wide variety of pathogens that are responsible for many common and some exotic infections. Widely used in animals.		
TRIMETHOPRIM, PLAIN **Huovinen P, Toivanen P. Trimethoprim resistance in Finland after five years' use of plain trimethoprim. Br Med J. 1980;280(6207):72-74.**	Antibiotic approved since the 1970's mostly used for the treatment of urinary tract infections.		2

Conclusion

The use of a longitudinal analysis with repeated measures is useful when looking at data through different points in time and when the descriptive statistics seem to be varied for all subjects in the dataset. In this case, the counties in South Carolina have different trends in the sales of antibiotics, have different population dynamics, and represent different spectrums for rate of premature death. The results of the analysis also indicate that higher antibiotic use continues to reflect a benefit for the population.

However, the seasonal analysis does reflect a change in behavior that is possibly not driven by bacterial infections, but by other factors unrelated to an appropriate use of antibiotics. If these trends continue, it is possible that those behaviors might contribute to antibiotic resistance. The different parts of this study may provide valuable information in the development and implementation of antibiotic stewardship programs that will be discussed in further detail in the next chapter. These analyses show how antibiotic use can certainly be the fuel for the Socio Ecological Coupling of Antibiotic Resistance.

CHAPTER 6:

DISCUSSION

Bacteria are able to acquire antibiotic resistance through natural processes and have done this throughout time in order to adapt to their changing environment. However over and incorrect use and disposal (such as in human waste, animal waste, or directly discharge) of antibiotics has contributed to the acceleration of bacteria of acquiring resistance. Determining where there is existing, or emerging, over or incorrect use of antibiotics in certain areas could help combat this acceleration. In the three parts of this study, different methods of understanding and researching antibiotic use and disposal are proposed.

Antibiotic use is affected based on geographic regions and population. There is significant difference from urban and rural areas in terms of total antibiotic use. However, in locations where there is a high deviation from the mean when considering time, these hotspots could be considered locations of interest for intervention. Some limitations from this study include not considering an age adjusted population and missing data for antibiotic use in animal operations. These two factors would strengthen the study, but as proposed in the research, they are also all part of the socio ecological coupling of antibiotic resistance and could be studied independently. In fact, research has been proposed that would study both these factors both independently and, later, their interactions.

To better understand the risk of human exposure to ARB in the environment, studies are needed at the intersection of the ARB socio-ecological cycle, where there is an increased potential for human exposure to ARB. This study was aimed at examining the generation of bioaerosols during the treatment of municipal wastewater as a possible route of ARB emission and an area of potential human exposure. Similar methods of wastewater treatment are prominent in urban settings throughout the developed and developing world, but the dispersal of ARB through bioaerosol generation has not been fully characterized. In this study, we first identified 42 different clinically relevant ARGs capable of conferring resistance to a range of antibiotics in the liquid obtained from the WWTP sludge aeration tanks. The most abundant mode of resistance conferred by the identified ARGs corresponded to the most prominently prescribed antibiotic classes (beta-lactams and fluoroquinolones) at the time of sampling within the WWTP's geographical location based on data from the CDDEP.

As demonstrated in Chapter 4, environmental surveillance is the only method that would give a more precise result on antibiotics being disposed, quantity of antibiotics present in the environment and, subsequently, antibiotic gene exchange rates and presence of antibiotic resistant bacteria. This study shows that environmental surveillance is costly and time demanding. It becomes complicated to reach a wide geographic area. In order to help reduce these demands and costs, more epidemiological studies are needed to develop a baseline and develop population focused indicators that could help target environmental surveillance.

As seen in Chapter 5, antibiotic use could be considered as an early indicator for premature death and quality of life in the population. The fact that there are seasonal cycles for antibiotic use demonstrates that there is a cause and a consequence of any difference in the number of prescriptions in a month or in a year; the difference will later be reflected in population health indicators. The epidemiological model presented shows a curve between antibiotic use and YPLL that is significantly affected by population. It also showed that this curve happens in every county in South Carolina independent of population changes. The results in Chapter 5 support the findings in Chapter 3, and add to the importance of mixed model studies, such as was done in this case by combining multilevel epidemiological methods with spatial analysis. These types of analysis are crucial support to environmental surveillance, to help identify hotspot locations for the type of research completed in Chapter 4.

For future research and other studies, it could be important to consider the rate of bacterial infections in the State and include them in the longitudinal study. The conclusion from Chapter 5 shows a protective effect from the increased use of antibiotics, or that antibiotics are still effective in clinical use, but it would be important to narrow the focus of the resaerch and look specifically at bacterial infections. It would also be relevant to compare the susceptibility of bacteria to the antibiotics through time.

Up for discussion is if these methods are part of antibiotic stewardship programs. The main goal of such stewardship programs is to protect patients from misdiagnoses, prevent the overuse of antibiotics, ensure the correct use of antibiotics, and effectively track antibiotic prescribing. Currently stewardship programs are becoming more

predominant, but are only part of major hospitals or health departments, not as part of broader public health policy. A stronger effort is necessary to reach the proposed goals by the Interagency Task Force for Combating Antibiotic-Resistant Bacteria created by CDC in 2013 (CDC, 2020). Data on antibiotic use should be more readily available and disclosure of effectiveness of antibiotics should be published. Antibiotics alone are clearly not the culprit in the cycle of antibiotic resistance. Less and less classes of antibiotics have been researched recently (Hutchings, et al. 2019) and stronger control on the misuse of antibiotics is necessary. All of this in an effort to prevent the antibiotic use vs. premature death curve (YPLL) to start shifting.

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