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Identifying Seasonal and Daily Variations in ARG-Containing Bioaerosols Generated During the Wastewater Treatment Process

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IDENTIFYING SEASONAL AND DAILY VARIATIONS IN ARG-CONTAINING BIOAEROSOLS GENERATED DURING THE WASTEWATER TREATMENT PROCESS

by

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DEDICATION

This work is dedicated to my parents who risked everything to move to the United States and give their children a shot at a better life and education.

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ABSTRACT

Antibiotic resistance is a growing problem with the current global death count topping 700,000. In the United States alone there are 2.8 million antibiotic resistant bacterial (ARB) infections each year and approximately 35,000 deaths. If current trends continue the global ARB death count will reach 10 million surpassing current chronic disease deaths. Wastewater treatment plants play a vital role in protecting both the environment as well as local communities. The WWTP process allows for the removal of chemicals and contaminants from water that eventually makes its way back into the environment as well as into drinking water plants. Despite the efficacy of the treatment process WWTPs have become reservoirs of antibiotic resistant bacteria. WWTPs function as a bridge between the sociological and ecological antibiotic resistant (AR) cycles so it is vital to investigate the fate of ARBs during the treatment process. Our data show that there is seasonal dependent variability in antibiotic resistant gene (ARG) abundance in aerosols generated during the WWTP process and that the warmer months experience a higher abundance of aerosolized ARGs as well as a higher variability in daily abundance. These data will be crucial in future work investigating the potential public health risk for exposure to aerosolized ARGs in WWTP employees and surrounding communities.

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LIST OF ABBREVIATIONS

AR ..... Antibiotic Resistance

ARB ..... Antibiotic Resistant Bacteria

ARG ..... Antibiotic Resistant Gene

BP ..... Base Pair

BOD ..... Biological Oxygen Demand

CDC ..... Centers for Disease Control

CI ..... Confidence Interval

DI ..... Deionized Water

EB ..... Elution Buffer

ORF ..... Open Reading Frame

PBS ..... Phosphate Buffered Saline

RR ..... Rate Ratio

WWTP ..... Wastewater Treatment Plant

CHAPTER 1

LITERATURE REVIEW

Antibiotics are the central focus and arguably a keystone in today's healthcare system and have been since the release of the first commercial antibiotic. These miracle drugs as they are sometimes coined are either naturally occurring or synthetic compounds. Initially the term antibiotic referred to the naturally occurring secondary metabolites produced by bacteria and fungi that possessed both growth inhibiting and killing potential (Nicolaou & Rigol, 2018). Their application spans human, animal and plant species and they are used for preventing and treating infections caused by pathogenic bacteria (Bouki et al., 2013). The first antibiotic to be discovered from nature was mycophenolic acid. In 1893 the Italian physician/microbiologist Bartolomeo Gosio isolated the antibiotic from Penicillium glaucum and discovered that the compound expressed antiviral, antifungal, antitumor, and anti-psoriasis properties. Unfortunately due to its publication in Italian , the discovery went unnoticed until it was rediscovered in the United States in 1913 (Nicolaou & Rigol, 2018). The most widely recognized antibiotic discovery is credited to Alexander Fleming with the discovery of penicillin. Fleming returned to his laboratory in September of 1928 to find a Staphylococcus aureus colony contaminated with Penicillium notatum. Unlike other scientists who disregarded this observation, Fleming performed a more in-depth investigation. After growing the fungus and using its extract to treat several pathogenic bacterial strains Fleming named the antibiotic penicillin in March of 1929 (Fleming, 1929).

After extensive use in the military during World War II, penicillin became commercially available to the public in 1945 ushering in a new age of medicine that has revolved around the discovery of new antibiotics (Nicolaou & Rigol, 2018; Pazda et al., 2019). Being described as the wonder drug, the discovery of penicillin led to increased research and discovery of more antibiotics such as tetracycline in 1948, vancomycin in 1958, methicillin in 1960, azithromycin in 1980, ciprofloxacin in 1987, daptomycin in 2003, etc.(CDC, 2019b). Many of the bacterial infectious diseases such as cholera, syphilis, plague, tuberculosis, or typhoid fever which would easily reach epidemic proportions before the twentieth century could now be easily treated with these new drugs (Mohr, 2016). Due to the popularity and widespread use of antibiotics, antimicrobial production has increased from 400 tons in the 1950s to over 15,800 tons in the late 1900s (Kim et al., 2007).

The main causes of antibiotic resistance are high use of antibiotics in agriculture, over prescription of antibiotics, longer that recommended treatment plans, inability to digest the antibiotics efficiently, not completing the antibiotic treatment, and improper disposal of antibiotics. All of these instances and bad practices result in large amounts of antibiotics being released into municipal wastewater (Gelband et al., 2015; Nagulapally et al., 2009). The CDC estimates that approximately 47 million antibiotics are prescribed each year for infections that do not require antibiotic treatment. This accounts for 30% of all antibiotic prescriptions. Additionally, nearly 70% of all prescriptions for sinus infections are longer than the recommended treatment, and between 2011-2016 there has only been a 5% decrease in antibiotic prescriptions (CDC, 2018). The number of antibiotic resistant bacteria (ARBs) is consistently increasing (Segura Pedro A. et al.,

2009) resulting in 671,689 infections and over 33,000 deaths in the European Union in 2015 (Cassini et al., 2019). According to the 2019 CDC report approximately 3 million infections in the United States are the result of ARBs and they accounted for almost 36,000 deaths in 2018 (CDC, 2019a). Additionally, one in five trips to the emergency room are due to the side effects of antibiotics (Naquin et al., 2015), and the annual healthcare costs due to ARB infections in 2014 was estimated to be 2.2 billion dollars annually (Thorpe et al., 2018).

As stated earlier, antibiotics are not fully digested in animals or humans. This results in approximately 30-90% of the consumed antibiotics being excreted through urine or feces (Gao et al., 2012). Once in the environment, antibiotics are not only able to exert toxin-like effects to bacteria, but are also able to influence selection pressure by existing in the environment at sub-inhibitory concentrations which leads to the proliferation of resistant bacterial cells that are immune to the effects of certain antibiotics (Birošová et al., 2014). Depending on the class of antibiotic, AR bacteria can exhibit four different methods of antimicrobial resistance including removing the antibiotic utilizing an efflux pump, creating an alternate metabolic pathway similar to the one inactivated by the antibiotic, modifying the antibiotic target, or deactivating the function of the antibiotic (Lin et al., 2015). The rapid spread and increase of antibiotic resistant bacteria can also be attributed to the variety of ways that antibiotic resistance genes (ARGs) are spread amongst bacterial colonies. In addition to vertical gene transfer (the transmission of genetic material to subsequent generations), bacteria can also utilize horizontal gene transfer including transformation, transduction, and conjugation to acquire new antibiotic resistant genes (Rizzo et al., 2013).

Since their inception in 1890, wastewater treatment plants (WWTPs) have played a vital role in the protection of the environment as well as the health of the public (Manaia et al., 2018). Initially, WWTPs were designed and built to remove debris, high organic loads, and pathogens from wastewater before being discharged into the environment (Henze et al., 2008). In today's society where population and urbanization is increasing rapidly, WWTPs acquire a large quantity of nutrients, metals, antibiotics, and chemicals from a variety of sources all of which couple with the ideal conditions in the treatment tanks such as temperature, stable pH, and close cell-to-cell interaction resulting in increased potential for horizontal gene transfer between bacteria (Karkman et al., 2018; Manaia et al., 2018; Naquin et al., 2015). Despite the advances in WWTP technology such as the separation of the process into stages that remove large contaminants as well as organic matter in the latter stages (Guardabassi et al., 2002) this process is not 100 percent effective (Giger et al., 2003). This results in effluent that is not truly sterile, but rather releases with it high amounts of bacteria that are of human or animal origin and harbors ARGs that have the potential to be disseminated back into the environment (Berendonk et al., 2015; Rizzo et al., 2013).

While the concentrations and effects of antibiotic resistant bacteria in aquatic environments is well known, there is a gap in knowledge in the effects of aerosolized AR pathogens. The term bioaerosol is used to describe viable and non-viable airborne biological particles such as fungal spores, bacteria, pollen and viruses as well as bacterial endotoxins, mycotoxins, and peptidoglycans. These particles have been found to make up a large portion of the atmosphere with some remote areas having 28% of their particulate matter comprise of bioaerosols. Additionally the largest concentration of microbes in the

air is situated directly above the ground surface during dry summers with moderate wind speeds (Korzeniewska, 2011). Wastewater treatment plants commonly use aeration tanks as part of their treatment process and since this step in the treatment process comes directly after the reception of the sewage influent, this project will investigate the seasonal differences in quantity and variate of AR pathogens being released into the environment surrounding the treatment tanks. Several studies have confirmed that the pretreatment, biological treatment, and sludge thickening processes (mixing, aerating, spraying, discharging) are responsible for the highest number of released bioaerosols and pathogens likely due to the mechanical nature of wastewater disturbance (Filipkowska et al., 2002; J. Li et al., 2016; Sánchez-Monedero et al., 2008). Additionally, a preliminary study by Gaviria-Figueroa et al., 2019 showed that bioaerosol samples collected downwind from liquid sludge tanks exhibited similar taxonomic profiles while samples collected upwind from the same tanks showed a distinct difference. We hypothesize that the abundance and the ARG profile will be greater and more diverse in the air surround the main treatment tanks during the warmer months when compared to areas further from the tanks and colder months.

CHAPTER 2

SEASONAL AND DAILY VARIATION IN ARG-CONTAINING BIOAEROSOLS

INTRODUCTION

While studies such as the ones conducted by (Filipkowska et al., 2002; J. Li et al., 2016) and Sánchez-Monedero et al., 2008 have shown that WWTPs emit bioaerosols throughout the treatment process, fewer studies have looked at the seasonal and daily variation in bioaerosols and fewer still have investigated the ARGs these bioaerosols carry. A study conducted in Turkey showed that there was a difference in bioaerosol levels in urban indoor environments between the winter and summer seasons (Mentese et al., 2012) while a study in China reported seasonal variability in airborne bacteria levels in an indoor WWTP (Ding et al., 2016). According to the preliminary ARG dispersal modeling done by Gaviria-Figueroa et al., 2019 ARG-containing bioaerosols at WWTPs have the potential to be carried several kilometers away from the source depending on wind speed. Therefore, it is important to understand the variability in ARG abundance over the course of all seasons.

MATERIALS AND METHODS

2.1. SAMPLE COLLECTION

The research site chosen for this project is the Columbia Metropolitan WWTP. The plant sits on 100 acres and serves approximately 60,000 customers over an area of

120 square miles. The plant was chosen because it employs two difference treatment technologies for sludge aeration; a bottom-injected air bubble aeration method as well as a surface mechanical aeration/agitation method. The project was performed over a span of one year to capture all four seasons (winter, spring, summer, fall) for investigation of potential temporal difference in ARG profiles throughout the year.

For each season, the sample collection spanned three consecutive days. The liquid sewage samples were collected in 50ml conical tubes from the influent tank, the bubble aeration tank, the surface agitation tank, and the effluent stream. The air samples were collected using SKC liquid impingers. The samplers were placed in an insulated tub and mounted onto a custom-built frame to simulate the average breathing zone (approximately 5'10"). Each stand contained three liquid impingers in order to collect the samples in triplicate. Two stands were placed at each of the three sites across the plant: the upwind site (location furthest from the treatment tanks), the bubble aeration tanks, and the surface agitation tanks. The liquid impingers contained 20ml of 0.5X phosphate buffered saline (PBS) and were attached to a vacuum pump that pulled 12.5 liters of air per minute per impinger and were run for six hours each day. The volume in the impingers was checked periodically over the course of the day and the PBS solution was adjusted with autoclaved DI water. Over the course of the six-hour sampling period 27,000 liters of air were filtered through the impingers at each site. At the end of each sampling day the PBS solution containing the bioaerosols was poured into 50ml conical tubes and stored on ice for transport back to the laboratory.

2.2. SAMPLE PROCESSING

In the lab, the liquid samples were vortexed for 30 seconds in order to homogenize the sample before being poured into 15ml tubes and centrifuged for 10 minutes at 4000xG. All but 1ml of the supernatant was removed and the sample was placed in a -80C freezer for storage until analysis. The air samples were processed in a similar fashion but were centrifuged for 20 minutes at 4000xG in order to ensure thorough pelleting of the sample. The samples were then taken through a DNA extraction process (Qiagen Powerviral DNA/RNA Kit, Hilden, Germany) as per the manufacturer's instructions and were eluted in 50 microliters of RNase-free water. The concentration of the samples was measured and recorded using a Qubit 2.0 (Life Technologies, Carlsbad, CA) before being used to prepare libraries for sequencing (New England Biolabs Ultra II FS DNA Library Prep Kit, Ipswich, MA). The samples were then combined in EB buffer and analyzed on a Bioanalyzer (Agilent, Santa Clara, CA) to ensure that the DNA had been fragmented to the appropriate size (~250bp) and that the concentration was approximately 15nM in 20ul. Samples were then sequenced using the Illumina NovaSeq 5000 platform (Illumina, San Diego, CA).

2.3. BIOINFORMATICS AND STATISTICAL ANALYSIS

Following sequencing, the raw DNA sequencing reads were first analyzed using the FastP quality control software (S. Chen et al., 2018)with the following settings [fastp -i inputfile_R1_001.fastq -I inputfile2_R2_001.fastq -o outputfile1_fastp.fastq -O outputfile2_fastp.fastq --unpaired1 filename_R12_fastp_unpaired.fastq --unpaired2 filename_R12_fastp_unpaired.fastq --failed_out filename_fastp_failed.fastq -Q -L -g --poly_g_min_len 5 --adapter_fasta adapterfiledirectory] in order to distinguish paired and

unpaired reads as well as trim poly-G tails which occur in two-color chemistry systems such as the NovaSeq. The cleaned sequences output from FastP were then processed through the SPAdes program (Nurk et al., 2013) for error correction using the following settings [spades.py --only-error-correction -m 800 -1 filename_R1_fastp.fastq -2 filename_R2_fastp_fastq -o filename_spades_error_corr] and the sequences assembled using Megahit (D. Li et al., 2015) with the following settings [megahit --presets meta-sensitive --min-contig-len 500 -1 filename_R1_fastp.00.0_0.cor.fastq.gz -2 filename_R2_fastp.00.0_0.cor.fastq.gz -r filename_R_unpaired.00.0_0.cor.gastq.gz -o outputfilename_over500_megahit]. After assembly, the contigs were analyzed using the Prodigal program (Hyatt et al., 2010) with the following settings [prodigal -i inputfile_final.contigs.fa -a filename_final.contigs_aa -d filename_final.contigs_nuc -f gff -o filename_final.contigs_gff -p meta] to predict open reading frames (ORFs). The Prodigal identified amino acid sequences were then aligned against the DeepARG antibiotic resistance gene database using DIAMOND (Arango-Argoty et al., 2018) with the following parameters [python /deepARG.py --align --genes --type prot --input filename_final.contigs_aa.fa --output filename_aa.fa.out]. The DeepARG data were then normalized using the following equation in order to make the metagenomes comparable (H. Chen et al., 2019):

$$\text{coverage}(\times/\text{Gb}) = \sum_{1}^{n} \frac{N_{mapped\ reads} \times L_{reads} / L_{ARG-like\ ORF}}{S}$$

where n is the number of annotated ARG-like ORFs belonging to that ARG type or subtype; $N_{mapped\ reads}$ is the number of the reads mapped to the ARG-like ORF; L_{reads} is the sequence length of Illumina reads; $L_{ARG\text{-}like\ ORF}$ is the length of the ARG-like ORF

sequence; *S* is the size of the data set (Gb). Finally, the data was graphed and analyzed using Tableau software. The data were plotted as normalized count data and the abundance of ARGs in the bioaerosol samples was averaged over daily triplicate measurements. A statistical analysis was performed using negative binomial regression with results expressed as rate ratios (RRs) with 95% confidence intervals (CIs). The RRs were checked for statistical significance using Wald test p-values with 95% CIs.

RESULTS AND DISCUSSION

2.4. TEMPORAL TRENDS IN ARG ABUNDANCE

Figure 2.1 shows the locations of the sampling sites at the WWTP as well as the variation in abundance in the air samples at the upwind and treatment tank sites over the course of the four seasons. The upwind air samplers were placed in the furthest possible location from the main treatment tanks in order to maintain an on-site control. At each sampling site the abundance of ARGs is higher during the spring and summer seasons, and Figure 2.2 shows that to be the case when the abundances for all sampling sites for each season are combined. Spring exhibited the highest abundance of ARGs with the summer and fall coming in at second and third respectively and the winter season having the lowest abundance of ARGs. Table 2.1 shows the statistical evidence for the patterns seen in Figures 2.1 and 2.2. Aerosolized ARG abundance was significantly lower in the winter than in any other season at the bubble aeration and surface agitation sites. The abundance of airborne ARGs at the bubble aeration and surface agitation tanks in the summer was over 7 times and 11 times higher respectively when compared to the abundance in the winter and over 8 times and 23 times higher in the spring respectively when compared to the abundance in the winter. The Wald p-test values were significant

across all subgroups, but the RRs and 95% CIs varied in value and range. Additionally, the ARG abundance in the aerosol samples at the upwind site was as low or lower than the aerosol samples at the bubble aeration and surface agitation sites during all seasons except for winter. The uncharacteristic result in the winter is due to the unusually high abundance value on day 3 for the upwind site. A potential reason for this uncharacteristic abundance is the wind patterns observed during the sampling day. With the various structures at the WWTP the air samplers may have been exposed to aerosolized ARGs originating from the treatment tanks.

In addition to the spring and summer seasons experiencing a higher abundance of ARGs, our data also show that during the warmer months the daily variation in ARG abundance was greater compared to the colder months (Fig. 2.3) indicating that there is a strong temperature dependent component to the patterns observed. While the average wind speed during our sampling days was slightly higher during the spring and summer seasons the increase in ARG abundance during these warmer months can be attributed to the increase in the observed temperature (Appendix Fig. 1). Higher temperatures often result in higher biological oxygen demand (BOD), and in order to meet this increased BOD the WWTP injects more oxygen into the treatment tanks which increases bacterial activity. This increase in wastewater agitation and microbial activity lends itself to the observed increase in aerosolized ARG abundance. The higher temperature coupled with wind speed could also be responsible for the higher variability in daily abundance in the warmer months. Our findings align with similar studies that looked at seasonal variability in bioaerosol emission. Both Ding et al., 2016 and Mentese et al., 2012 observed higher airborne bacteria counts in the summer season when compared to the winter season.

Table 2.1. Estimated RRs with 95% CIs and Wald test p-values from negative binomial regression analyses for seasonal comparisons of total ARG abundance by sampling site. Statistically significant results have been bolded.

Sampling site	Comparison	RR	95%	CI	Wald test p-value
All bacterial classes combined					
Bubble sludge	Spring vs. Winter	1.65	0.58	4.65	0.3471
	Summer vs. Winter	0.87	0.31	2.47	0.7977
	Fall vs. Winter	2.93	1.04	8.28	0.0423
Surface sludge	Spring vs. Winter	0.96	0.22	4.23	0.9598
	Summer vs. Winter	2.45	0.56	10.7	0.2354
	Fall vs. Winter	0.83	0.19	3.65	0.8074
Bubble aeration	**Spring vs. Winter**	**8.34**	**3.04**	**22.9**	**0.0000**
	Summer vs. Winter	**7.93**	**2.89**	**21.7**	**0.0001**
	Fall vs. Winter	**6.35**	**2.31**	**17.4**	**0.0003**
Surface agitation	**Spring vs. Winter**	**23.25**	**9.96**	**54.2**	**0.0000**
	Summer vs. Winter	**11.51**	**4.93**	**26.8**	**0.0000**
	Fall vs. Winter	**7.18**	**3.08**	**16.7**	**0.0000**
Upwind	Spring vs. Winter	2.88	0.62	13.2	0.1748
	Summer vs. Winter	1.50	0.33	6.94	0.6003
	Fall vs. Winter	0.24	0.05	1.12	0.0692
All bacterial classes except glycopeptide-resistant bacteria					
Bubble sludge	Spring vs. Winter	1.58	0.51	4.85	0.4265
	Summer vs. Winter	0.94	0.30	2.88	0.9073
	Fall vs. Winter	3.19	1.04	9.82	0.0427
Surface sludge	Spring vs. Winter	0.87	0.20	3.79	0.8515
	Summer vs. Winter	2.14	0.49	9.33	0.3107
	Fall vs. Winter	0.60	0.14	2.62	0.4978
Bubble aeration	Spring vs. Winter	6.82	1.83	25.4	0.0042
	Summer vs. Winter	6.36	1.71	23.7	0.0059
	Fall vs. Winter	4.60	1.23	17.1	0.0231
Surface agitation	Spring vs. Winter	13.71	6.73	27.9	0.0000
	Summer vs. Winter	7.05	3.46	14.3	0.0000
	Fall vs. Winter	5.32	2.61	10.8	0.0000
Upwind	Spring vs. Winter	2.76	0.73	10.4	0.1348
	Summer vs. Winter	1.78	0.47	6.75	0.3938
	Fall vs. Winter	0.19	0.05	0.73	0.0158
Glycopeptide-resistant bacteria only					
Bubble sludge	Spring vs. Winter	1.67	0.61	4.59	0.3182
	Summer vs. Winter	0.85	0.31	2.33	0.7492
	Fall vs. Winter	2.83	1.03	7.77	0.0436
Surface sludge	Spring vs. Winter	1.02	0.23	4.56	0.9780
	Summer vs. Winter	2.64	0.59	11.8	0.2033

Sampling site	Comparison	RR	95%	CI	Wald test p-value
	Fall vs. Winter	0.98	0.22	4.36	0.9758
Bubble aeration	Spring vs. Winter	10.26	4.62	22.7	0.0000
	Summer vs. Winter	9.91	4.46	22.0	0.0000
	Fall vs. Winter	8.54	3.84	18.9	0.0000
Surface agitation	Spring vs. Winter	32.23	12.49	83.1	0.0000
	Summer vs. Winter	15.72	6.09	40.5	0.0000
	Fall vs. Winter	8.93	3.46	23.0	0.0000
Upwind	Spring vs. Winter	2.98	0.54	16.3	0.2096
	Summer vs. Winter	1.29	0.23	7.06	0.7726
	Fall vs. Winter	0.28	0.05	1.54	0.1432
Multidrug-resistant bacteria only					
Bubble sludge	Spring vs. Winter	1.85	0.63	5.38	0.2608
	Summer vs. Winter	0.90	0.31	2.63	0.8521
	Fall vs. Winter	3.46	1.19	10.0	0.0230
Surface sludge	Spring vs. Winter	0.99	0.21	4.58	0.9914
	Summer vs. Winter	1.95	0.42	8.99	0.3930
	Fall vs. Winter	0.59	0.13	2.74	0.5031
Bubble aeration	Spring vs. Winter	6.78	1.75	26.3	0.0056
	Summer vs. Winter	7.12	1.84	27.6	0.0045
	Fall vs. Winter	4.76	1.23	18.4	0.0241
Surface agitation	Spring vs. Winter	15.92	7.43	34.1	0.0000
	Summer vs. Winter	8.37	3.91	17.9	0.0000
	Fall vs. Winter	4.67	2.18	10.0	0.0001
Upwind	Spring vs. Winter	3.25	0.81	13.0	0.0962
	Summer vs. Winter	2.27	0.57	9.11	0.2469
	Fall vs. Winter	0.20	0.05	0.79	0.0223

Figure 2.1. Map of sampling sites at Metro WWTP with ARG abundance (normalized count) for each seasonal time point in 2019 (BA=Bubble Aeration; SA=Surface Agitation).

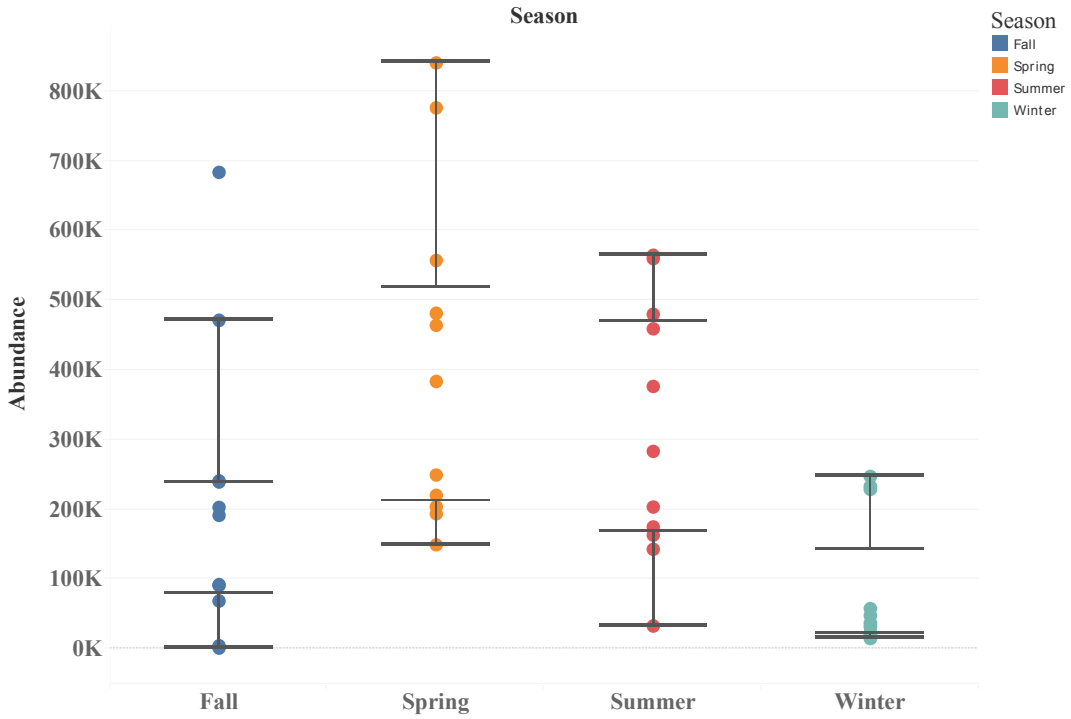

Figure 2.2. Total abundance (normalized count) of ARGs found in combined liquid and air samples collected at the Metro WWTP across all seasonal time points.

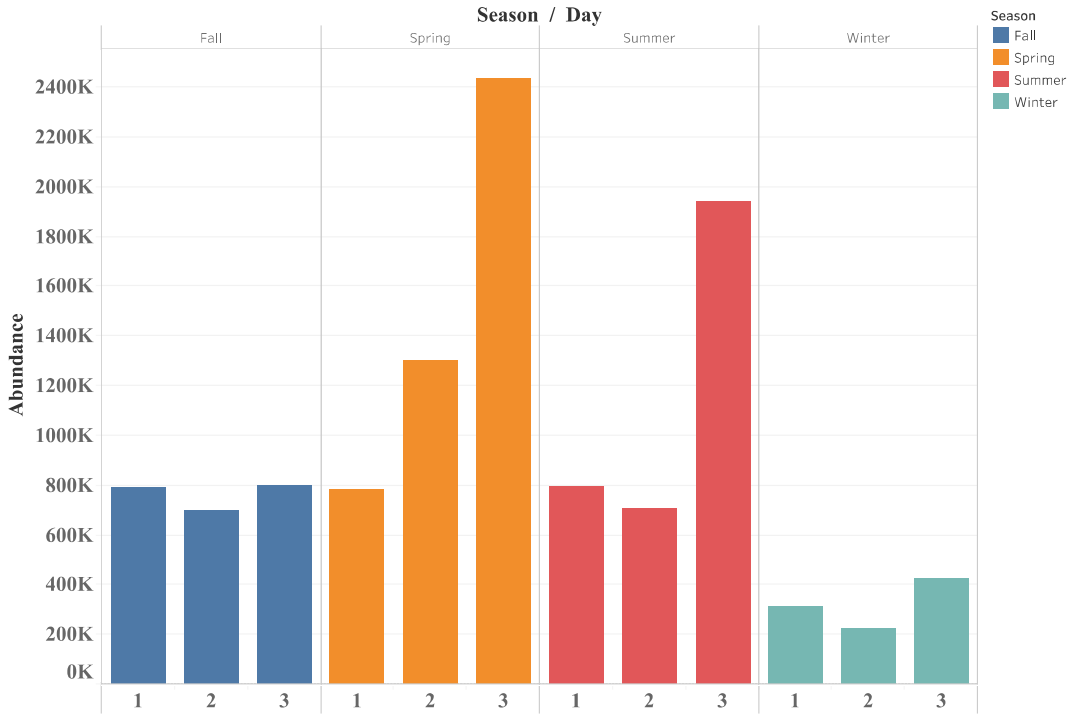

Figure 2.3. Daily variation in total (liquid and air) ARG abundance (normalized count) across all seasonal time points.

2.5. SEASONAL ARG PROFILE COMPARISON

Despite the variation in ARG abundance between sites and seasons, our data show that the highest number of genes collected during each season were genes that confer resistance to the glycopeptide family of antibiotics (vancomycin, teicoplanin, telavancin, etc.). Additionally, Fig. 2.4 shows that genes that confer multidrug resistance were second highest in abundance across all seasons followed by unclassified ARGs. When the ARG abundance is compared across sampling sites (Fig. 2.5) the pattern seen in Fig. 2.4 is still present. Glycopeptide ARGs are the most abundant across all sites and seasons followed by multidrug and unclassified ARGs.

For the statistical analyses, glycopeptide-resistant bacteria, multidrug resistant bacteria, and unclassified bacteria were treated as separate classes while the remaining classes were combined. Table 2.2 shows that across all four seasons (with all sampling sites combined), the abundance for ARGs conferring glycopeptide resistance was significantly higher than the other classes. When compared to multidrug resistant ARGs, glycopeptide-resistant ARG abundance was almost four times higher across all four seasons. Additionally, when compared to the unclassified ARGs and the remaining combined classes the ARG abundance for glycopeptide-resistant bacteria was more than 7 times higher and more than 100 times higher respectively. When the abundance counts were combined over all seasons and evaluated by sampling site, abundance for glycopeptide-resistant ARGs was still statistically significantly more abundant than all other classes. Excluding the glycopeptide vs. multidrug comparison at the upwind site, all of the Wald p-test values were statistically significant. However, the 95% CIs were wide suggesting that statistical power may have been too low.

Table 2.3 shows that the ARG abundance for multidrug resistant ARGs was significantly higher (more than 25 times as abundant) than the remaining ARG classes across all four seasons as well as at all sampling sites. All of the 95% CIs were very wide however indicating low statistical power. When comparing unclassified ARGs to the remaining ARG classes the abundance was at least five times higher across all four seasons. Unclassified ARG abundance was also significantly higher than the remaining ARG classes at each sampling site. All of the Wald p-test values were statistically significant however the 95% CIs were very wide. The sludge source material for both bubble aeration and surface agitation were mostly similar in ARG abundance across the four seasons. While the RRs showed that the ARG abundance in bubble aeration sludge was higher in the spring and fall and that the ARG abundance in surface agitation sludge was higher in the summer the Wald p-test showed that the RRs were statistically non-significant. This proved to be true for all four class-based subgroups (Table 2.4).

Table 2.2. Estimated RRs with 95% CIs and Wald test p-values from negative binomial regression analyses for comparisons of total abundance between glycopeptide-resistant ARGs and other ARG classes, by season or sampling site.

[a] "Remaining" stands for bacterial classes other than glycopeptide, multidrug or unclassified, combined
[b] All sampling sites combined
[c] All seasons combined

Comparison	Season or sampling site	RR	95% CI	Wald test p-value
Glycopeptide vs. multidrug	Spring[b]	3.86	2.20 - 6.76	0.0000
	Summer[b]	3.68	1.82 - 7.47	0.0003
	Fall[b]	4.60	1.55 - 13.64	0.0058
	Winter[b]	3.91	1.51 - 10.10	0.0048
	Bubble sludge[c]	5.49	2.46 - 12.27	0.0000
	Surface sludge[c]	4.86	2.08 - 11.34	0.0003
	Bubble aeration[c]	2.39	1.07 - 5.33	0.0335
	Surface agitation[c]	4.67	1.59 - 13.72	0.0051
	Upwind[c]	2.13	0.76 - 6.00	0.1529
Glycopeptide vs. unclassified	Spring[b]	23.48	13.40 - 41.15	0.0000
	Summer[b]	13.60	6.71 - 27.59	0.0000
	Fall[b]	18.34	6.19 - 54.31	0.0000
	Winter[b]	7.48	2.90 - 19.33	0.0000
	Bubble sludge[c]	17.02	7.61 - 38.05	0.0000
	Surface sludge[c]	10.66	4.57 - 24.90	0.0000
	Bubble aeration[c]	11.99	5.37 - 26.76	0.0000
	Surface agitation[c]	27.65	9.41 - 81.28	0.0000
	Upwind[c]	15.37	5.45 - 43.32	0.0000
Glycopeptide vs. "remaining" [a]	Spring[b]	126.3	72.00 - 221.62	0.0000
	Summer[b]	118.6	58.44 - 240.89	0.0000
	Fall[b]	117.8	39.77 - 349.28	0.0000
	Winter[b]	129.7	50.13 - 335.89	0.0000
	Bubble sludge[c]	144.0	64.32 - 322.44	0.0000
	Surface sludge[c]	123.4	52.80 - 288.62	0.0000
	Bubble aeration[c]	88.98	39.83 - 198.77	0.0000
	Surface agitation[c]	136.5	46.43 - 401.58	0.0000
	Upwind[c]	93.88	33.26 - 264.96	0.0000

Table 2.3. Estimated RRs with 95% CIs and Wald test p-values from negative binomial regression analyses, for comparison of total abundance between multidrug-resistant or unclassified ARGs and the "remaining" ARG classes combined, by season or sampling site.

[a] "Remaining" stands for bacterial classes other than glycopeptide, multidrug or unclassified, combined
[b] All sampling sites combined
[c] All seasons combined

Comparison	Season or sampling site	RR	95% CI	Wald test p-value
Multidrug vs. "remaining" [a]	Spring[b]	32.74	18.66 - 57.44	0.0000
	Summer[b]	32.22	15.87 - 65.41	0.0000
	Fall[b]	25.60	8.64 - 75.86	0.0000
	Winter[b]	33.18	12.82 - 85.88	0.0000
	Bubble sludge[c]	26.23	11.72 - 58.74	0.0000
	Surface sludge[c]	25.42	10.87 - 59.44	0.0000
	Bubble aeration[c]	37.25	16.68 - 82.23	0.0000
	Surface agitation[c]	29.24	9.94 - 86.01	0.0000
	Upwind[c]	44.10	15.63 - 124.46	0.0000
Unclassified vs. "remaining" [a]	Spring[b]	5.38	3.07 - 9.44	0.0000
	Summer[b]	8.72	4.29 - 17.71	0.0000
	Fall[b]	6.43	2.17 - 19.05	0.0008
	Winter[b]	17.34	6.70 - 44.89	0.0000
	Bubble sludge[c]	8.46	3.78 - 18.95	0.0000
	Surface sludge[c]	11.58	4.95 - 27.07	0.0000
	Bubble aeration[c]	7.42	3.32 - 16.58	0.0000
	Surface agitation[c]	4.94	1.68 - 14.52	0.0037
	Upwind[c]	6.11	2.16 - 17.24	0.0006

Table 2.4. Estimated RRs with 95% CIs and Wald test p-values from negative binomial regression analyses for comparisons of total ARG abundance between bubble aeration and surface agitation sludge by season.

Season	RR (bubble vs. surface sludge)	95% CI		Wald test p-value
All bacterial classes combined				
Spring	1.74	0.52	5.80	0.3650
Summer	0.36	0.12	1.09	0.0698
Fall	3.59	0.88	14.66	0.0748
Winter	1.02	0.33	3.12	0.9729
All bacterial classes except glycopeptide-resistant bacteria				
Spring	1.37	0.42	4.54	0.6026
Summer	0.33	0.10	1.15	0.0815
Fall	4.02	1.09	14.77	0.0361
Winter	0.76	0.24	2.34	0.6286
Glycopeptide-resistant bacteria only				
Spring	1.94	0.57	6.63	0.2911
Summer	0.38	0.14	1.05	0.0609
Fall	3.43	0.79	14.85	0.0997
Winter	1.18	0.35	3.99	0.7853
Multidrug-resistant bacteria only				
Spring	1.38	0.43	4.46	0.5899
Summer	0.34	0.09	1.32	0.1193
Fall	4.32	1.16	16.16	0.0295
Winter	0.74	0.23	2.36	0.6123

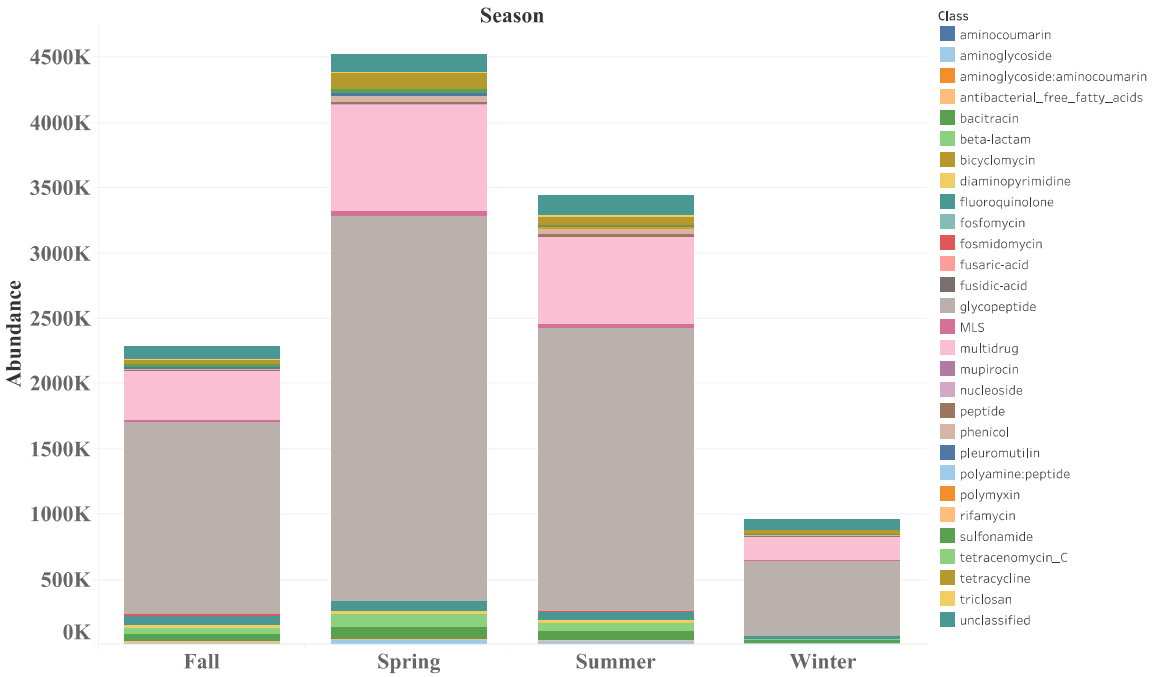

Figure 2.4. Abundance of ARGs across all seasonal time points in 2019 by antibiotic class.

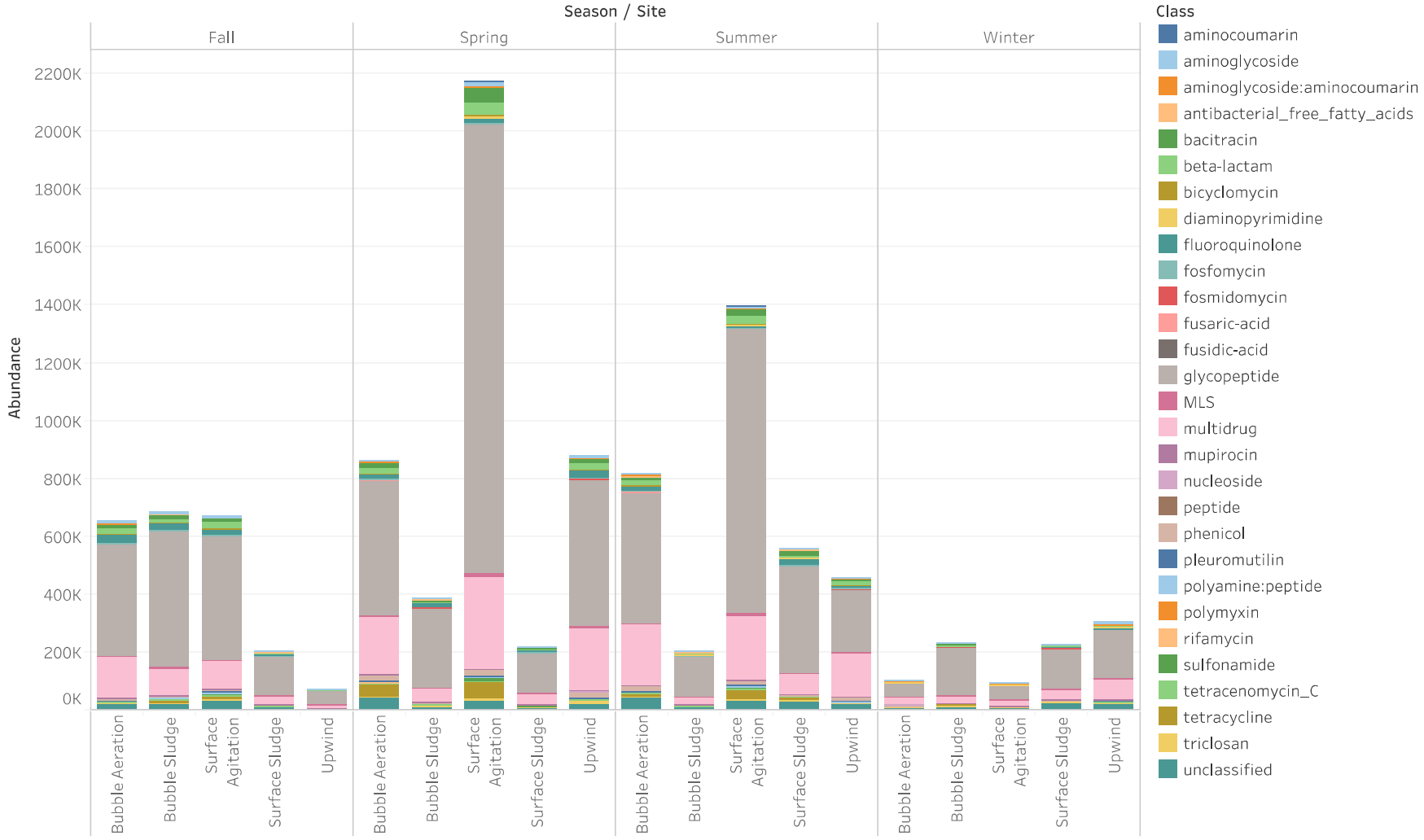

Figure 2.5. Abundance of ARGs across all seasonal time points and sampling sites in 2019 by antibiotic class.

CHAPTER 3

FUTURE DIRECTIONS

The dangers of antibiotic resistance cannot be overstated. With millions of people becoming infected with ARBs and tens of thousands of people dying each year in the United States alone it is imperative to understand the fate of antibiotic resistant bacteria in the environment. In addition to the samples taken at the Metro WWTP, samples were also collected at the WWTPs in Charleston as well as nasal, sputum, and stool samples from WWTP employees that volunteered to be a part of the study. That data will be used to investigate the differences in treatment technologies within and between the WWTPs as well as identify any potential risks that WWTP employees may be exposed to from aerosolized ARGs. The identified ARGs will also be analyzed at the gene level, taxonomically classified and identified for any pathogens of concern. Additionally, with the emergence of SARS-CoV-2 and the resulting pandemic, liquid samples from the treatment tanks are being collected at the Metro WWTP in order to monitor and identify a potential increase in antibiotic use and subsequently antibiotic resistant bacteria. This work will be vital in protecting the health of the public by identifying any potential for exposure to the communities surrounding wastewater treatment plants and will assist the treatment facilities in decisions regarding any design changes that can reduce the potential exposure.

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APPENDIX A

SEASONAL TEMPERATURE AND WIND SPEED METADATA

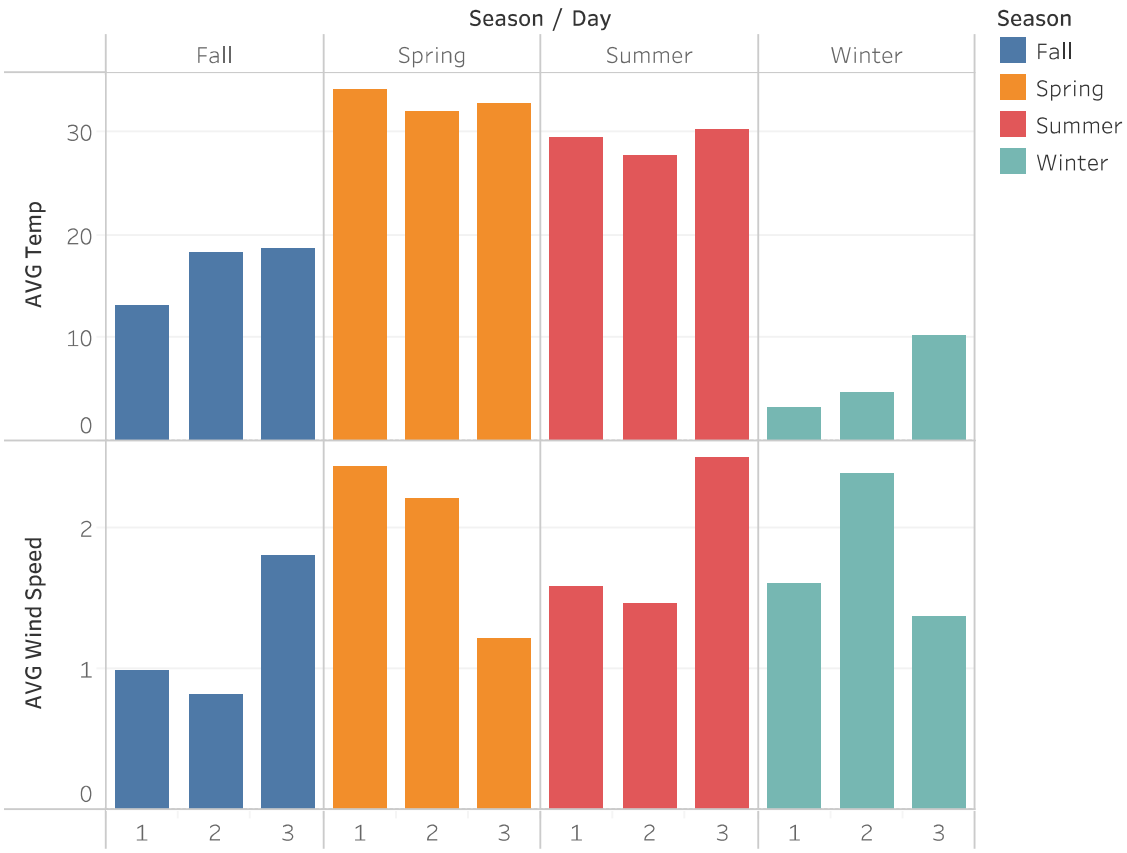

Figure A.1. Average daily temperature and wind speed for each sampling day in 2019.