AN INTEGRATIVE APPROACH TO IDENTIFY AND ASSESS STREAMS SUSCEPTIBLE TO ANTIBIOTIC DISCHARGES

by

Sara Kamanmalek

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Approved by:

____
Dr. Jacelyn Rice-Boayue

____
Dr. Sandra Clinton

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Dr. Oyla Keen

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Dr. Mariya Munir

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Dr. Wenwu Tang

ABSTRACT

SARA KAMANMALEK. An Integrative Approach to Identify and Assess Streams Susceptible to Antibiotic Discharges. (Under the direction of DR. JACELYN RICE-BOAYUE)

Occurrences of antibiotics and antibiotic resistance have been reported in various environmental settings, posing a global concern due to associated human and ecological risks. Therefore, the main objective of this study is to develop a holistic approach to determine the most-impacted streams and watersheds to antibiotics and antibiotic resistance across the U.S. Wastewater treatment plant (WWTP) effluent is a major point source of antibiotics and antibiotic resistance in the aquatic environment. Thus, WWTPs and receiving streams were integrated into a GIS-based model to assess the impacts of WWTP effluent and influencing factors such as instream flow, WWTP effluent flow rates, and estimated environmental concentrations on antibiotic resistance threats at the national level. Concentrations of commonly detected antibiotics such as ciprofloxacin, erythromycin, and sulfamethoxazole were predicted at WWTP discharge sites. Predicted concentrations were then compared into the antibiotic resistance safety threshold calculated from the predicted no-effect concentrations (PNECs) for selection resistance to determine streams that are susceptible to the potential presence of antibiotic resistance. Then, we examined the geospatial distribution of watershed vulnerability to antibiotic resistance contamination by the development of a multimetric index considering multiple point and nonpoint pollution sources. In addition to WWTPs, other antibiotic point sources (hospitals and total antibiotic prescription) and nonpoint sources (antibiotic use by food-producing animals and manure application) were incorporated into the model as well as dam storage ratio as the hydrologic indicator and projected minimum temperature change as a climate change indicator. Consequently, the index of watershed vulnerability to antibiotic resistance was calculated, which ranks most to least resistance-impacted watersheds. Lastly, outcomes of the previous parts of the study were translated into targeted field analysis quantifying selected antibiotics within NC watersheds that are modeled to be most impacted by antibiotic pollution sources. In addition, we used hot spots analysis to determine counties with intense hot spots of antibiotic-impacted watersheds and investigate the racial and socioeconomic status of identified counties in NC. This study presented a holistic approach to assess spatial hazards of antibiotics and antibiotic resistance, and such information can be used to prioritize watershed management, control, and mitigation strategies in impacted watersheds.

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DEDICATION

To my beloved parents. Thank you for your unconditional love and support.

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7Q10	7-day 10-year low flow
ARGs	Antibiotic resistance genes
ARB	Antibiotic resistance bacteria
ABR	Antibiotic resistance
AMR	Antibiotic microbial resistance
AOPs	Advanced oxidation processes
AR	Antibiotic resistance
AWaRe	Access, Watch, and Reserve
AZI	Azithromycin
CAFOs	Concentrated animal feeding operation
CECs	As contaminants of emerging concern
CFS	Cubic feet per second
CIP	Ciprofloxacin
CMIP5	Coupled model intercomparison project
CWNS	Clean watersheds need survey
DDD	Defined daily dose
DEM	Digital elevation model
DFs	Dilution factors
DO	Dissolved oxygen
DRINCS	De facto reuse incidence nations consumable supply
DWTPs	Drinking water treatment plants
EECs	Estimated environmental concentrations
ERY	Erythromycin
FDA	The Food and Drug Administration
GIS	Geographic information systems
HIFLD	Homeland Infrastructure Foundation-Level data
HLB	Hydrophilic-lipophilic-balance
HQ	Hazard quotient
HUC	Hydrological unit code

IWI	Index of watershed integrity
Koc	The organic carbon/water partition coefficient
LA-MRSA	Livestock-associated *methicillin-resistant Staphylococcus aureus*
LC-MS	Liquid chromatography-tandem mass spectrometry
LOC	Level of concern
LOD	Level of detection limit
LULC	Land-use and cover
MBR	Membrane bioreactor
MECs	Measured environmental concentrations
MGD	Million gallons per day
MMI	Multi-metric index
NCCV	National climate change viewer
NE	Northeast
NHDPlus	National hydrography data set plus
NID	National Inventory of Dams
NRSA	National Rivers and Streams Assessment
PCS	Permit compliance system
PNECs	Predicted no-effect concentrations
QA/QC	Quality assurance and quality control
RCP	Representative concentration pathway
REF	Reference
SES	Socioeconomic status
SMX	Sulfamethoxazole
SPE	Solid-phase extraction
SRT	Solids retention time
SSO	Strahler stream order
SWAT	Soil and Water Assessment
TRI	Trimethoprim
USEPA	U.S. environmental protection agency

USGS	United states geological survey
UV	Ultraviolet
VRE	Vancomycin-resistant enterococci
WBD	Watershed boundary dataset
WHO	World health organization
WQI	Water quality index
WWTPs	Wastewater treatment plants

CHAPTER 1: INTRODUCTION

1.1 ANTIBIOTICS AND ANTIBIOTIC RESISTANCE IN SURFACE WATER

Anthropogenic sources of antibiotics and antibiotic resistance in surface water include untreated and treated municipal wastewater, treated sewage sludge (biosolids), sewage from hospital and pharmaceutical manufacturing facilities, agriculture, and aquaculture. High antibiotic concentrations have been detected within treated effluent or/and downstream of the WWTP discharge point. Sources of antibiotic resistant bacteria and genes in surface water have been tracked to close by WWTPs[1, 2]. Biosolids contain elevated concentrations of antibiotics such as ciprofloxacin and azithromycin[3] and several antibiotic resistance genes (ARGs) encoding resistance to multiple classes of antibiotics[4], which can enter the soil/groundwater system when applied as agricultural fertilizer. Extensive use of antibiotics to promote animal growth and treat infections in concentrated animal feeding operations (CAFOs) coupled with incomplete metabolism results in a growing concern regarding the spread of antibiotics and antibiotic resistance in manure and manure-treated lands and thus surface water[5]. Hospital sewage is expected to be enriched in ARGs and antibiotic residue due to the high consumption rate in patients[6]. Antibiotic residues have been detected at higher concentrations in pharmaceutical manufacturing facilities sewage than hospital sewage[7].

Substantial use of antibiotics, incomplete metabolism, and removal coupled with the lack of regulations, discharge requirements, and surveillance monitoring results in the detection of antibiotic residues and antibiotic resistance in surface water[8, 9]. Previous studies have assessed antibiotic and antibiotic resistance in surface water mainly based on monitoring approaches by investigating the correlation between a limited number of antibiotics and the corresponding genes[1,7]. However, field studies are restricted by cost and time constraints impacting sample size with site selection mostly dependent on local knowledge of the area of interest. Risk assessment, exposure quantification, and potential control and mitigation strategies can be aided by an integrative approach characterizing possible key sources of antibiotics and antibiotic resistance and their respective contributions and variability[10]. Therefore, this study suggests an integrative framework that provides a cost-effective assessment of antibiotics and antibiotic resistance occurrence at national and regional levels through the linkage of anthropogenic activities across the watershed scale, which was used to inform the design of a field study quantifying antibiotics in most impacted watersheds within North Carolina.

1.2 OBJECTIVES OF THE STUDY

The main goal of this study is to identify watersheds that are more vulnerable to antibiotic resistance due to elevated antibiotic inputs from potential nonpoint and point antibiotic pollution across the U.S. One of the major point sources of antibiotics and antibiotic resistance in surface water has proven to be municipal WWTP discharge. To identify to what extent WWTP discharge contributes to antibiotic concentrations and potential resistance presence, a GIS-based model was utilized to assess antibiotic concentrations at the national level, followed by the integration of antibiotic PNECs for selection resistance. The influence of key factors on the model performance including instream flow conditions, WWTP effluent flow, and estimated environmental concentrations of antibiotics was investigated. The next step is to assess watershed vulnerability to antibiotic resistance contamination by the development of the multimetric index that incorporates potential point and nonpoint antibiotic sources, hydrologic conditions, and climate change. Lastly, the developed multimetric index was downscaled and adapted to identify the most impacted watershed by antibiotic pollution sources in North Carolina to be further investigated with field study. The objectives of the dissertation were:

1. To quantify antibiotic concentrations at WWTP discharge sites and to identify streams more susceptible to antibiotic resistance across the U.S.
2. To identify watershed vulnerability to antibiotic resistance due to cumulative impacts of antibiotic point and nonpoint pollution from CAFOs, manure application, WWTPs, hospitals, and antibiotic use patterns across the U.S.
3. To conduct a targeted field study quantifying selected antibiotics within three North Carolina watersheds that are modeled to be most impacted by potential sources (i.e., WWTPs and CAFOs).

1.3 SIGNIFICANCE OF THE STUDY

The intellectual merit of this study lies in the utilization of a holistic time and cost-effective modeling approach to identify vulnerable watersheds to potential AR contamination across the U.S. through the integration of large datasets. This project shows that environmental modeling can be coupled with sampling and field analysis to detect contaminant hotspots, prioritize adoption of advanced treatment processes, and monitoring in at-risk watersheds. Implementation of this approach can be done in an effort to minimize the negative effects of antibiotics and ARGs on ecological health.

Outcomes of the models can be used to inform decision-making and policy development at the national and state levels. In addition, the model can be expanded in the future to investigate the effect of additional factors or sources of antibiotics on potential antibiotic presence in surface water. The project's findings will be broadly disseminated in the form of three journal articles and an online mapping that presents data, datasets, and outcomes of the model. The resulting online mapping will provide insight into regional variations across the U.S. and will support the identification of future field studies for contaminant occurrence analysis.

1.4 DISSERTATION ORGANIZATION

This dissertation consists of six chapters. Chapter 1 is an introductory chapter providing an overview of antibiotic and antibiotic resistance occurrences in the aquatic environment and the approach taken in this study. Chapter 2 provides a relevant literature review on antibiotics and antibiotic resistance in surface water and factors impacting the development and dissemination of antibiotic resistance as well as gaps in the literature. Chapters 3, 4, and 5 are presented in form of research papers addressing gaps and research questions. Chapter 3, "Spatial Hazards of Antibiotic Resistance in Wastewater Impacted Streams During Low Instream Flow Conditions", has been submitted in the journal of *ACS ES&T Water*. Chapters 4, "Geospatial Distribution of Watershed Vulnerability to Antibiotic Resistance in Streams Across the U.S.", is in preparation for submission to the journal of *Science of the Total Environment*. Chapter 5, "An Integrative Assessment of Antibiotic Concentrations in North Carolina Watersheds", is in preparation for submission in *Environmental Research*. Summary of the findings, conclusions, and recommended future works are provided in Chapter 6.

CHAPTER 2: BACKGROUND

2.1 MAJOR SOURCE CONTRIBUTORS TO ANTIBIOTICS AND ANTIBIOTIC RESISTANCE IN THE AQUATIC ENVIRONMENT

Antibiotics and antibiotic resistance (AR) can enter the environment through several pathways that stem from human and animal consumption of antibiotics. Major point and nonpoint source contributors include wastewater treatment plants (WWTPs), concentrated animal feeding operations (CAFOs), aquaculture operations, medical care facilities, and pharmaceutical manufacturing facilities[11]. Antibiotic use and prescription rates have been positively correlated with antibiotic resistance in receiving anthropogenically impacted watersheds[11-13]. The Food and Drug Administration (FDA) has reported that 11.5 million kgs of antibiotics were sold or distributed in animal food-producing farms in 2019, with 6.2 million kgs classified as medically important antibiotics (i.e., common among humans and animals)[14]. Due to extensive use and incomplete metabolism, animal manure contains antibiotics and AR residues which can end up in the aquatic environment by agricultural runoff from animal farms and manure-treated lands[15-17]. Aquaculture effluent is another major source contributor of antibiotic resistance to the environment[18-20]. In regard to human prescription use, FDA reports indicate that more than 3.3 million kgs of antibiotics were sold internally in 2011 across the U.S., primarily sold in pharmacy settings[21]. Extremely high concentrations of antibiotics and antibiotic resistance have been reported in the effluent of pharmaceutical manufacturers [22, 23] and hospitals[24, 25]. Municipal WWTPs may treat sewage that has accumulated from domestic households, hospitals, and pharmaceuticals. This is due to most hospital and pharmaceutical facilities discharging wastes into the municipal sewer system. Antibiotics can also spread through soil and ultimately contaminate groundwater by binding to biosolids when sewage sludge is applied as an agricultural fertilizer[26, 27].

2.1.1 OCCURRENCE, FATE AND TRANSPORT OF ANTIBIOTICS AND ANTIBIOTIC RESISTANCE IN WWTPs

Antibiotics, antibiotic resistance genes (ARGs), and antibiotic-resistant bacteria (ARB) have been commonly reported in the effluent of municipal WWTPs, posing ecological risks on receiving water bodies[28-30]. The most commonly detected antibiotics in municipal WWTP effluent are the classes of quinolones (ciprofloxacin and ofloxacin), tetracyclines, sulfonamides (sulfamethoxazole), trimethoprim, and macrolides (azithromycin, erythromycin/erythromycin-H2O, and roxithromycin), where the most frequently reported ARGs from WWTP effluent are *tet* (*tetQ*, *tetO*, *tetW*), *sul* (*sul1*, *sul2*), *ermB*, and *bla* (bla_{CTXM}, bla_{TEM})[31]. For example, the median of measured antibiotic concentrations within effluent across the U.S. for azithromycin, trimethoprim, ciprofloxacin, erythromycin, and sulfamethoxazole are 230.5, 98.0, 193.2, 107.0, and 385.0 ng /L, respectively (*Appendix 8.1*, Table A9 and [32, 33]). ARG concentrations downstream of WWTPs have been significantly higher than upstream and have shown a positive correlation to distance from discharge points[34, 35]. To illustrate, the concentration of Class 1 integron (*intl1*) was 20 times higher in receiving water body compared to upstream of WWTP in the Texas area, highlighting WWTP contribution to the spread of ARGs in the aquatic environment[34].

Different types of treatment technologies employed by WWTPs can decrease antibiotic and ARB concentrations and ARG enrichment in the treated municipal discharge to different degrees. Primary treatment has been documented to be more efficient in tetracycline and chlortetracycline removal than sulfamethoxazole and erythromycin[36, 37]. Stronger Van der Waals forces and interactions between electron donor and acceptor may be associated with increased tetracycline elimination by adsorption to solids[38]. Antibiotic removal efficiency varies significantly in conventional secondary treatment. Physical and chemical properties of antibiotics and environmental conditions play a significant role in the capability of secondary treatment to remove antibioics[39, 40]. Although conventional secondary treatment is not designed to remove antibiotics, removal of a few antibiotics has been observed[36, 41-44]. Sulfamethoxazole and trimethoprim rates of removal efficiency during biological treatment were 68% and 20%, respectively[36]. Since the sorption of sulfonamides and trimethoprim is minimal, microbial degradation is the main removal mechanism during secondary treatment[41]. Slow-growing nitrifying bacteria play a primary role in the biodegradation of trimethoprim [42]. Thus, an increase in solids retention time (SRT) from 60 to 80 days significantly increases trimethoprim removal efficiency[36]. Complete removal of tetracycline and chlortetracycline has been reported during secondary treatment[36]. The removal mechanism for tetracyclines is most likely sorption to sludge due to their poor biodegradability[43, 44]. Accumulated antibiotics in sludge and biosolids can pose risks if proper treatment is not applied to waste prior to land application[45].

Advanced treatment generally has shown higher antibiotic removal rates. According to the 2012 Clean Watersheds Need Survey (CWNS), 13,245 WWTPs across the U.S. discharge treated effluent to surface water, while 5,384 WWTPs have implemented advanced treatment technologies including chlorination and ultraviolet (UV) which are more frequently implemented as well as less frequent technologies such as granular and powdered activated carbon, reverse osmosis, membrane processes, and ozonation[46]. Between chlorination and UV irradiation which are the most frequently implemented advanced treatment technologies in WWTPs across the U.S., chlorination has shown a higher removal efficiency for commonly prescribed antibiotics. For example, some antibiotic removal efficiencies via chlorination include: ofloxacin (100%), ciprofloxacin (100%), tetracycline (99%), erythromycin (98%), trimethoprim (93%), sulfamethoxazole (81%), sulfamethazine (80%), chlortetracycline (78%), doxycycline (67%)[37, 47-52]. Li and Zhang (2011) have compared antibiotic concentrations within secondary and tertiary effluent of the same WWTP[53], obtaining average removal efficiencies of 62% for sulfamethoxazole, 26% for erythromycin, 55% for ciprofloxacin, 42% for trimethoprim in the activated sludge process. Additionally, they reported removal efficiencies in the chlorination process and total removal efficiencies of 27% and 73% for sulfamethoxazole, 24% and 43% for erythromycin, 18% and 66% for ciprofloxacin, and 40% and 65% for trimethoprim, respectively. As a result, using chlorination yielded higher removal efficiencies of antibiotics in the final effluent for sulfamethoxazole by 11%, erythromycin by 17%, ciprofloxacin by 11%, and trimethoprim by 23%[53]. Generally, chlorination is significantly effective for the removal of sulfonamides, macrolides, and tetracyclines[41].

In general UV irradiation is significantly less effective in antibiotic removal than chlorination with an inconsistent removal rate due to different light sensitivity of antibiotics in the influent, short UV exposure time, and limited choices of wavelength. Yang et al (2014) concluded that UV irradiation at dosages applied in the WWTP disinfection process is ineffective in antibiotic removal[54]. UV irradiation has removed macrolides with a removal efficiency of 15%, while chlorination removal efficiency was reported at 45%[55]. In addition, a bench-scale study indicated that UV irradiation alone results in insignificant macrolides removal, even at higher UV doses[48]. After a 10-minute exposure, UV irradiation removed trimethoprim with an efficiency of only 7%[47]. Lin and Tsai (2009) reported that UV irradiation yielded 66% tetracycline removal, a lower removal efficiency than chlorination (90%)[51].

Other tertiary treatment technologies have been documented as efficient in antibiotic removal; however, they are not frequently applied to full-scale WWTPs[46]. Ozonation is reported to be efficient in the removal of commonly prescribed antibiotics with 40%-80% removal efficiency[56]. Activated carbon resulted in 60% sulfamethoxazole removal from secondary effluent[57]. Complete removal of trimethoprim and sulfamethoxazole has been achieved using chlorinated reverse osmosis following secondary effluent[58]. In comparison to secondary treatment, a combination of sand filtration and ozonation increased the removal efficiency of sulfamethoxazole from 38% to 98%, trimethoprim from 35% to 99%, and ofloxacin from 61% to 85%[59]. Most antibiotics have been fully removed using a combination of UV irradiation with neutral photo-Fenton ($Fe^{2+,3+}$/H2O2/$UV_{254,}$ and $Fe^{2+,3+}$/H2O2/simulated sunlight)[47].

Resistance genes encoding different groups of antibiotics have been detected in secondary treatment processes[60], the most widely employed system in the U.S. (two-third of WWTPs)[61]. While some studies reported that the activated sludge process significantly decreases the number of ARGs[62, 63], others reported the enrichment of some ARGs in the treated effluent[64-66]. For example, the enrichment of certain resistance genes including *tetB, bla*$_{CTX\text{-}M}$, and *dfrA3* was observed in the effluent of a conventional WWTP[66].

Despite the reduction in the number of bacteria even up to 99% by wastewater treatment processes, enrichment of ARGs in the effluent was observed, showing the different dynamics of ARGs from the fate of bacteria[64, 67, 68]. Consequently, the spread of antibiotic resistance can possibly be promoted by WWTPs employing solely biological processes[29].

Recent studies show that higher levels of treatment decrease the potential threats of ARB, including advanced oxidation processes (Fenton oxidation)[69], conventional disinfectants (ozone and chlorine)[70], UV treatment[71, 72], or a combination of the[73] due to the disturbance in bacterial cells. However, their effectiveness in ARG reduction in full-scale WWTPs is negligible. For example, UV treatment was able to remove ARB with the removal efficiencies in the range of 34-75% at the UV dose of 27 mJ/cm^2 [71], while a significant reduction in ARGs has not occurred at the same UV dose[26, 74]. Munir et al. (2011) observed that a UV dose of 27 mJ/cm^2 is incapable of the significant reduction in *tetW*, *tetO*, and *sulI* gene abundance in full-scale WWTPs and reported that a much higher UV dose was required to reduce ARGs[26]. According to McKinney and Pruden (2012), a UV dose of 200-400 mJ/cm significantly reduced ARGs in filtered wastewater[75]. Chlorination was unable to effectively remove ARGs from wastewater by detailed qPCR examination of tetracycline and erythromycin resistance genes[70]. Chlorination yielded a reduction in the absolute abundance of ARGs by 89.0%–99.8%; however, significant ARG levels were detected in WWTP effluent, up to $9.5 \pm 1.8 \times 10^5$ copies/mL[76]. Zhang et al. (2017) observed a positive relationship between ARG removal efficiencies, a higher dosage of disinfectant (chlorine), and longer contact/exposure time, where free chlorine dosage of 30 mg/L results in maximum ARG removal[73]. In addition, their results showed that chlorination was generally more effective in ARG removal compared to UV[73]. Additionally, high efficiency in ARG removal was achieved using UV irradiation followed by chlorination[73]. Despite the complete inactivation of antibiotic-resistant cultivable bacteria by the integration of solar Fenton oxidation into a membrane bioreactor (MBR), ARGs including *ampC*, *sulI*, and *ermB* were still detected in the effluent[69]. Although promising, removal efficiency of ARGs and ARB by disinfection processes such as chlorination, ozonation, and UV irradiation were achieved in the lab and pilot-scale, further studies are needed to tackle issues found in regard to the efficiency of disinfection processes in the removal of ARGs and ARB in full-scale WWTPs.

2.1.2 HOSPITAL ANTIBIOTIC CONTRIBUTIONS TO MUNICIPAL WWTP FLOW

In addition to municipal wastewater, hospital wastewater has been identified as a significant source of antibiotic contamination in the environment[77]. Due to its specific nature, hospital effluent is likely to discharge a combination of compounds (e.g. antibiotics, pathogens, and resistance) into WWTPs, contributing to the spread of ARGs in surface water[78, 79]. Several monitoring studies reported elevated levels of antibiotic resistance in hospital wastewater[80, 81]. For example, antibiotic-resistant pathogens such as Vancomycin-resistant enterococci (VRE), *E. coli* carrying ESBL, and the resistant opportunistic pathogen *P.aeruginosa* have been found to be enriched in hospital effluents[82, 83]. Ofloxacin and ciprofloxacin were present in hospital effluent near the Ter River in Spain, at concentrations of over 13 μg/L[84].

Nevertheless, there is limited research regarding the contribution of hospital effluents towards antibiotic and antibiotic resistance loading in WWTPs[85-89]. While some studies have found hospital effluent to be a significant, yet not unique, source of antimicrobial residues and ARBs in WWTPs[90-93], others have observed no consistent effect of hospital effluent on the development and persistence of ARGs within WWTPs[11, 94-98]. Hospitals commonly discharge their effluents directly into the sewer system [79]. The co-treatment practice of hospital effluents and domestic wastewater in municipal WWTPs has been questioned by some researchers suggesting the implementation of a more unique pretreatment of hospital effluents before being discharged into municipal WWTPs[89, 99] in order to avoid environmental losses due to sewer overflow and leakage. In addition to avoiding the dilution of domestic wastewater with hospital effluent, which can inhibit biomass growth, leading to a reduction of removal efficiency in WWTPs[100].

Prior work has analyzed the contribution of hospitals and WWTPs to antibiotic and ARG presence in effluent-impacted streams by investigating the correlation between the concentration of some selected antibiotics and the occurrences of corresponding ARGs in WWTP effluent and downstream of receiving rivers on a site by site basis[79, 90, 94]. However, this approach is restricted by the availability of suitable sampling sites, especially due to the inaccessible and complex sewer system near hospitals[101]. In addition, the measured concentrations cannot be transferred to other locations as a meaningful representative of antibiotic and ARG concentrations to assess hospital and WWTP contribution unless there is a thorough knowledge of the system regarding the hospital audit data and WWTP's flow[102]. This highlights the need for an approach towards assessing hospital contributions to WWTPs. Data on hospital wastewater flow and municipal WWTPs receiving hospital discharges are, however, still not available, limiting the current assessment of the emission of antibiotic compounds from hospitals[103]. To tackle this challenge, we propose to improve the DRINCs model by spatially linking hospital discharges to WWTPs and estimating hospital discharge flow from typical water usage values per bed. Consequently, such a methodology will serve as a tool to determine whether hospital effluent should be prioritized over other significant sources of antibiotic prevalence in the environment.

2.1.3 CAFO CONTRIBUTIONS TO ANTIBIOTIC RESISTANCE

Antibiotics have been widely used to inhibit and control disease outbreaks in livestock and treat infectious disease[104]. Antibiotic use to boost animal growth is banned in the EU since 2006[105]; However, countries including the U.S. still use antibiotics in sub-therapeutic doses as growth promoters. However, this practice in the U.S. is restricted to non-medically important antimicrobials since 2017[106]. Despite the extensive use of antibiotics in livestock, reliable datasets regarding quantity and antibiotic use patterns including frequency and dose are not available[107]; Therefore, quantification of antibiotic use in the livestock industry is a challenging task[107]. According to the Food and Drug Administration (FDA) reports on 2019 sales and distribution of "medically important antimicrobials" (i.e., also used in the treatment of humans), tetracycline accounted for the largest volume of sales (4.1 million kg, 41% of total sales). In addition, an estimated 42%, 41%, 10%, and 3% of medically important antimicrobials are expected to be used in swine, cattle, turkeys, and chickens, respectively[14]. ARG abundance in livestock waste indicates a significant difference among different types of livestock. Greater ARG abundance and diversity have been detected in swine and chicken manure than cow and fish[108, 109].

Livestock has been identified as one of the main sources of antimicrobial resistance in the environment due to antibiotic overuse and misuse[110]. Antibiotic resistance developed in CAFOs can be transmitted directly or indirectly to humans, other animals, and the environment via the food chain, air, water, sludge-fertilized, and manured soils.[111-113]. Surface streams near CAFO farms have been reported to have a high level of antibiotic resistance[114]. For example, West et al. (2010) noted the highest level of multi-drug-resistant bacteria at sites located in the proximity of CAFOs; higher resistance than downstream of WWTPs[114]. In addition, higher percentages of tetracycline- and erythromycin-resistant enterococci were detected in surface water downgradient from CAFO (swine farm)[115].

Previous studies investigated whether the relationship between exposure to CAFO and environmental justice variables (i.e., percentage of low-income and minority population) exists across the US[116-118]. A study on the location of CAFOs concerning poverty and race in adjacent Census block groups in North Carolina, the second-largest state for hog industry, found that census blocks with higher percentages of low-income communities and minorities have disproportionately higher exposure to CAFOs, indicating environmental disparities regarding socioeconomic status (SES) and race in NC[116]. Another study conducted in Mississippi also indicated that CAFOs are disproportionately situated in regions with high numbers of African Americans and people living in poverty[118]. These studies suggest the potential contribution of environmental disparities from CAFO-related exposure to racial and socioeconomic disparities in health. Previous studies have been focused on the impact of CAFOs on the occurrence of antibiotic resistance in watersheds by conducting field studies on a small geographical scale. However, little work has been done on investigating the comparative contribution of CAFOs on antibiotic resistance content in the watershed at the national scale. To this end, we incorporated antibiotic use by food-producing animals into the spatial-based model to determine watershed vulnerability to AR occurrences in the U.S.

2.2 FATE, TRANSPORT, AND GEOGRAPHIC LOADING VARIATIONS OF ANTIBIOTICS

Antibiotic concentrations are highly variable in different geographical regions around the world, especially due to differences in consumption rate[119]. World Health Organization (WHO) has reported antibiotic consumption from 2016 to 2018 for 65 countries located in 6 regions worldwide in an effort to facilitate the development of strategies combating antimicrobial resistance[120]. Counties with the highest consumption rate per region include the United Republic of Tanzania (Africa), Brazil (America), Turkey (Europe), Iran (Eastern Mediterranean Region), and Mongolia (Western Pacific Region) with defined daily dose (DDD) per 1000 inhabitants per day of 27.29, 22.75, 38.18, 38.78 and 64.41, respectively. In addition, the WHO report also indicates the consumption rate for antibiotic class characterized by WHO's AWaRe categories (Access, Watch, and Reserve). More than 50% of antibiotic use belongs to the Access group which includes antibiotics suggested as a first or second choice due to the wide range of applications and lower resistance potential, with the highest consumption rate of amoxicillin[120]. Therefore, antibiotics belonging to Access are expected to have higher occurrences in surface water such as tetracycline, trimethoprim, sulfamethoxazole/ trimethoprim, and doxycycline. The Watch group includes most of the highest priority antibiotics with a relatively high risk of selection of bacterial resistance such as azithromycin, ciprofloxacin, and erythromycin, which accounts for 20% to 50% of consumption in reported countires[120]. WHO recommends prioritization of monitoring and stewardship programs for antibiotics in the Watch group. Reserve group accounts for less than 2% of antibiotic consumption which their use should be tailored to "high priority" pathogens and multidrug-resistant infections[120]. Although the WHO report lacks some countries such as China and U.S., DDD for antibiotics per 1000 inhabitants per day for these countries are reported as 157 and 28.8 in the literature, respectively[121].

Antibiotic concentrations in Asian developing countries were substantially greater than in North American and European countries due to the larger population and easier accessibility of over-the-counter antibiotics, leading to higher consumption rates and measured levels in the environment [122]. For example, ciprofloxacin was reported at significantly high levels near an industrial Indian site, Patancheru, at concentrations up to 14,000 µg/L in WWTP effluent, 6,500 µg/L in Lake, and 14 µg/L in groundwater, while ciprofloxacin has been detected at ng/L in surface waters across European Union and Northern America[123, 124]. In addition, ofloxacin and trimethoprim were reported up to 160 and 4.4 µg/L in the effluent of WWTPs in Patancheru[22, 125]. These quantities observed in India are among the highest ever detected. Additionally, High concentrations of antibiotics have been reported in other Asian countries including South Korea[126], China[127], and Pakistan[125].

Geospatially distributed antibiotic loadings within WWTP effluent provide information regarding regions with potential hazards of ARGs. Rodriguez-Mozaz et al. (2015) reported mean concentrations of ciprofloxacin, cefotaxime, and trimethoprim at 139.3, 220.17, and 109.9 ng/L in the effluent of 3 WWTPs discharging into Ter River in Spain[84]. The highest concentrations of penicillin G[128] and penicillin V[129] were reported up to 300 and 2,000 ng/L, respectively, in WWTP effluent in Australia. Amoxicillin and erythromycin-H2O were determined in quantities of up to 1,670 and 4,330 ng/L in Hong Kong[130]. The highest concentration of erythromycin detected in the effluent is 620 ng/L in Germany[131]. Data for ciprofloxacin indicated a concentration of up to 5,600 ng/L in WWTP effluent in the U.S[132]. Kelly and Brooks (2018) and Schafhauser et al. (2018) examined the global occurrence of ciprofloxacin and erythromycin in several different water matrixes including municipal WWTP effluent[32, 33]. Global statistics for erythromycin and ciprofloxacin concentrations are 196 and 188 ng/L as median; and 2,500 and 1,0530 ng/L as 90th percentile of MECs in WWTP effluent, respectively[32, 33]. U.S. data for ciprofloxacin indicated 193 ng/L as the median and 8,745 ng/L as the 90th percentile of MECs in WWTP effluent, whereas the MECs of erythromycin were 107 and 332 ng/L as the median and the 90th percentile, respectively[33].

Understanding the degradation mechanism for each antibiotic plays an important role in implementing effective treatment technologies and antibiotic fate in the natural environment. Photodegradation is a dominant degradation pathway for most antibiotics in the environment[125, 133-136]. The composition of the liquid matrix (dissolved organic content, pH, chloride ion concentration) contributes significantly to the observed photodegradation[137]. Mobility is an important factor in the degradation mechanism of antibiotics. As an example, trimethoprim and sulfonamides are expected to have high mobility based on estimated soil organic carbon-water partitioning coefficient (Koc), while fluoroquinolones, tetracyclines, and erythromycin are expected to have low mobility, leading to adsorption to the sediments and suspended solids in the aquatic environment[119, 138]. Koc values for sulfamethoxazole and trimethoprim are estimated at 72 and 75, respectively, whereas Koc values at much higher quantities are predicted at

570 for erythromycin, 61,000 for ciprofloxacin, 100,865 for amoxicillin, and 44,143 for ofloxacin [138]. The antibiotic stability in the aquatic ecosystem determines whether antibiotic concentrations persist. For example, due to relatively low environmental stability, oxytetracycline was rarely detected in WWTP effluent and surface water[139], with concentrations lower than the limit of detection for most of the studies conducted in North American surface waters[140-142]. Additionally, despite the excessive use of beta-lactams, especially penicillin, instability of the beta-lactam ring and its susceptibility to hydrolysis results in rare detection of these antibiotics in WWTP effluent[143-145]. Byproducts of antibiotic degradation can vary by antibiotic compound and treatment process. An increase in sulfate concentration was observed during the degradation of sulfathiazole by photolysis[136, 146]. Similarly, the formation of ions such as sulfate and nitrate were observed as the degradation byproducts of antibiotics containing nitrogen and sulfur in their structures using advanced oxidation processes (AOPs) [147].

2.3 POTENTIAL ECOLOGICAL IMPACTS FROM ANTIBIOTIC RESISTANCE

At low concentrations, antibiotics have long-term and short-term impacts on environmental health, including alterations to microbial biodiversity and functions and the development of ARB and ARGs [148-150]. The presence of antibiotics can impose constant selection pressure on bacterial populations resulting in antibiotic microbial resistance (AMR) being carried by ARB and expressed through ARGs[151-153]. The evolutionary dynamics in microbial populations can be impacted by selective pressure imposed by the antibiotic presence in different ways[151]. Fluctuations in critical physiological traits or gene expression changes can cause different tolerance levels to antibiotics in certain bacterial populations [154, 155]. While strong selective pressure can favor the growth of resistant microbial lineages resulting in diversity reduction in the microbial population, weak selective pressure can increase diversity by selectively favoring the growth of bacterial lineages[151]. For example, a larger colony size in *Staphylococcus* spp. was correlated with intermediate concentrations of antibiotics such as ciprofloxacin, streptomycin, and amikacin due to higher adaptability and genetic diversity of different types of bacteria [156-158]. In addition, exposure to different levels of antibiotics was found to diversify the microbial population genetics,[159, 160] to horizontal gene transfer between bacteria [161, 162], and to impact gene regulation at the transcription level[163, 164].

The performance of important ecological functions including decomposition, primary productivity, and nutrient cycling are highly affected by bacteria and fungi inhabiting the aquatic environment [165-167]. Selective pressures associated with antibiotic concentrations in environments can reduce taxa diversity or impose a shift in the microbial composition, altering the overall microbial community and causing a loss of microbial taxa, which perform critical ecological roles[168]. For example, overall microbial diversity, including taxa performing primary productivity and carbon cycling, was reduced by the antibiotic presence in the aquatic environment [169-171]. Denitrification was found to be limited due to the delay in the start of cell growth caused by exposure to sulfamethoxazole at a concentration as low as 0.005 μM [172]. Changes in the bacterial community under long term antibiotic stresses were found in the river receiving municipal effluent from two antibiotic manufacturing sites, including a decrease in bacterial community similarities, abundance in *Deltaproteobacteria*, *Clostridia*, and *Bacilli* in the presence of oxytetracycline, and abundance in *Epsilonproteobacteria* in the presence of Penicillin G [173]. Due to the negative ecological impacts of antibiotics and ARGs, the quantification of antibiotics in surface water gives an insight into the vulnerability of streams and watersheds to potential ARG presence.

2.4 CONTRIBUTION OF GEOGRAPHIC INFORMATION SYSTEMS ON HYDROLOGIC AND WATER QUALITY MODELING

Water resource studies have benefited from the use of Geographic Information Systems (GIS). For over two decades, GIS has been used in hydrologic modeling to perform pre- and post-processing on spatial hydrologic modeling data, directly enabling spatial analysis and modeling.[174]. The hydrologic analysis benefits from utilizing GIS, by improved accuracy and product complexity, greater efficiency, timeliness, ease of data sharing, more flexibility, more accessible map storage, and less duplication[175]. Spatial relationships can integrate qualitative and quantitative data instead of an attribute that may not be an accurate representation[176]. Visualizing data through these spatial relationships is possible which is one of the most appealing features in GIS and can enhance decision making[177, 178].

GIS has been used in many empirical studies investigating hydrologic problems. Prior studies have been conducted in the following areas: determination of hydrologic parameters, hydrologic assessment, hydrologic modeling inside GIS for steady-state processes, and hydrologic model set up[179]. As an example, GIS has been used in other hydrologic research, including stormwater[180] and groundwater modeling[181], flood risk assessments[182]; point and nonpoint pollution; water quality studies[183, 184], surface run-off estimation[185], and hydrological drainage modeling[186] at different geographical scales from regional watershed to global water resource planning[187, 188]. Wan et al. (2013)

incorporated the land-use and cover (LULC) into climate change to predict the effect on the Wolf Bay watershed's streamflow in southern Alabama using the Soil and Water Assessment (SWAT) Tool [189]. In general, assessment of point and nonpoint source pollution on small watersheds and large river basins can be done by simulating major hydrologic components and interactions by SWAT. In addition, iSTREEM is an online GIS-based model that estimates contaminant concentration in stream networks across the U.S by incorporating WWTPs, level of treatment, hydrologic data, drinking water treatment plants (DWTPs), and instream decay rate of the contaminant[190]. A combination of field study and water quality modeling has been integrated into the GIS framework to examine the spatial distribution of water quality in the Nile River by building the mathematical model of Water Quality Index (WQI)[191].

Modeling studies have incorporated hydrologic modeling into GIS to understand the fate and effects of antibiotics and antibiotic resistance in the environment. A study conducted in China investigated emissions and multimedia (water, air, soil) fate of frequently detected antibiotics using human and animal consumption data and level III fugacity model[121]. Using a similar modeling approach, Johnson et al. (2015) assessed antibiotic concentrations and risk of toxicity in European rivers using only human consumption data, exertion rate, and WWTP removal efficiency. Modeled antibiotic concentrations from both studies were comparable to measured environmental concentrations (MECs) of selected antibiotics, indicating the effectiveness of the modeling framework in predicting antibiotic concentrations and potential antibiotic resistance presence[192]. In addition, cluster analysis of AMR possible sources and transmission routes within the GIS framework has been used to select sampling locations and it also improves improve policy and monitoring systems[193]. The work performed here utilized the unique capabilities offered by GIS to assess the potential for antibiotic resistance occurs within a watershed under varying hydrologic conditions.

2.5 CLIMATE CHANGE EFFECTS ON STREAMFLOW AND CONTAMINATION CONCENTRATIONS IN SURFACE WATER

Streamflow significantly affects water quality parameters and contaminant concentrations in surface water. For example, high seasonal streamflow during the wet season yielded lower antibiotic concentrations in an urban river due to higher dilution[194]. This diluting effect of the wet season on contaminant concentrations is expected downstream of point contributors (i.e WWTPs, hospitals, etc)[9, 194]. Contrastingly, the highest antibiotic concentrations have been detected downstream of CAFOs during the wet season due to higher surface runoff compared to the dry season[195]. Therefore, antibiotic and ARG concentrations downstream of nonpoint contributors (i.e., CAFOs, manure-applied agricultural land, etc) are expected to increase during the wet season. Drought condition leads to lower dilution factors in effluent-impacted streams[196] elevating exposure duration and antibiotic resistance[197]. In addition, increases in ambient temperature have increased bacterial growth rate [198] and horizontal gene transfer (i.e., the main mechanism in the dissemination of antibiotic resistance)[199]. For example, MacFadden et al. (2018) investigated the impact of local temperature (average minimum temperature) on antibiotic resistance across the U.S., which noted positive associations between the increasing local temperature on antibiotic resistance[200].

To predict the effect of climate change on streamflow, both numerical and imperial models have been routinely used in many regions across the U.S.[201] Regional variations in temperature and streamflow due to climate change are less certain primarily due to uncertainties in the prediction of the spatial pattern[196]. However, a decline in streamflow at the regional level has been predicted by many studies. For example, lower mean annual streamflow has been modeled for the Rocky Mountain region (especially Rocky River with more frequent drought) [202] [203, 204], parts of Pacific (Sacramento-San Joaquin) [204, 205], parts of Southwest (the Rio Grande and Arizona) [204, 206] and Northeast and Midwest U.S.[207]

The minimum temperature will increase across the U.S. based on USGS National Climate Change Viewer (NCCV). The minimum temperature projection is derived from 30 CMIP5 climate models downscaled to an 800-m grid for the RCP8.5 and RCP4.5 emissions scenarios[208]. The highest projection changes in minimum temperature are predicted for northern parts of North Dakota, Minnesota, and parts of Utah, Idaho, and Wyoming. Therefore, the work performed here incorporated projected minimum temperature change as a climate change indicator into the multi-metric index identifying watershed vulnerability to AR occurrences.

2.6 SUMMARY OF RESEARCH NEEDS

To increase the understanding of antibiotics and antibiotic resistance occurrences, a holistic approach identifying point and nonpoint sources (i.e., WWTPs, hospitals, pharmaceutical production facilities, CAFOs, manure, etc.) and their respective contribution is required. In addition, the impacts of hospitals and health care facilities on municipal wastewater systems need to be investigated. Research is required to assess seasonal and climate-driven instream flow conditions and their impact on the antibiotic and resistance concentrations downstream of sources, especially point sources such as

WWTPs. This study developed an integrative approach to identify and assess watersheds susceptible to antibiotic resistance at the national scale. Firstly, we evaluated the impact of WWTPs on antibiotic concentrations in receiving streams by developing a GIS model that incorporates nationwide WWTPs to stream flowlines. In doing so, selected antibiotic concentrations were quantified downstream of WWTPs under varying streamflow conditions including, mean annual, mean monthly, and 7-day 10-year (7Q10) low flow conditions, and were then compared to antibiotic resistance safety threshold to identify Wastewater-impacted streams that are more prone to antibiotic resistance hazards. Secondly, we determined the geospatial distributions watershed vulnerability to antibiotic resistance occurrences by point and nonpoint pollution. Lastly, the nationwide model was downscaled to the state of North Carolina and used to perform an integrative assessment, where model predictions were used to identify the top three most impacted watersheds by antibiotic pollution to be further investigated by field study.

CHAPTER 3: SPATIAL HAZARDS OF ANTIBIOTIC RESISTANCE IN WASTEWATER IMPACTED STREAMS DURING LOW INSTREAM FLOW CONDITIONS

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3.1 ABSTRACT

Wastewater treatment plants (WWTPs) are a major point source of antibiotics and antimicrobial resistance in the aquatic environment [1, 2]. This study evaluated concentrations of ciprofloxacin, erythromycin, and sulfamethoxazole, and the potential presence of their respective resistance within U.S. streams affected by WWTP discharges under varying instream flow conditions. We incorporate predicted no-effect concentrations for selection resistance into previously developed De Facto Reuse Incidence Nations Consumable Supply (DRINCS) model to identify potential antibiotic hotspots across the U.S. Our results suggest that under mean annual instream flow, more than one-third of sites (*4,629 out of 13,245*) did not meet antibiotic resistance (ABR) safety threshold for ciprofloxacin. Under low instream flow conditions, dilution factors in 76.9% (*n=9,885*) of sites exceeded the ABR safety threshold for ciprofloxacin, and ABR safety thresholds for two antibiotics were surpassed in 25.8% (*n=3,323*) of streams with available low flow data (*n=12,856*). Despite considerable therapeutic use and the resulting presence of sulfamethoxazole in the effluent, the threat of sulfamethoxazole resistance is comparatively low due to a higher ABR threshold. We suggest that several streams across the U.S. are vulnerable to antibiotic resistance development from compounds with relatively low PNECs, such as ciprofloxacin, during average and low instream flow conditions.

3.2 INTRODUCTION

Global antibiotic consumption and incomplete metabolism, paired with partial removal in wastewater treatment plants (WWTPs) result in detection in the aquatic environment at the nanogram to low microgram per liter concentrations range[3]. WWTPs have been documented as one of the main point sources of antibiotics and antimicrobial resistance within aquatic ecosystems [1, 2]. U.S. Environmental Protection Agency and U.S. Geological Survey conducted five national studies to measure antibiotics in several environmental settings, including streams[4], groundwater[5], ground- and surface water source of drinking water[6], sewage sludge from publicity owned plants[7] and WWTPs discharge [8]. Antibiotics were present in all five studies. For example, sulfamethoxazole and erythromycin have been detected in 12.5% and 21.2% of 104 streams across 30 states, at maximum concentrations of 1,900 and 1,700 ng/L, and median concentrations of 150 ng/L and 1000 ng/L, respectively.

At low concentrations, antibiotics can elicit impacts on the environment by altering biodiversity and ecological functions, and further present public health risks corresponding to the development of antibiotic resistance [9, 10]. The antibiotic presence may complicate the presence of antibiotic resistance in the environment by posing constant selective pressure on the bacterial population resulting in the enrichment of antibiotic resistance bacteria (ARB) as well as facilitating the spread of antibiotic resistance genes (ARGs) through the horizontal gene transfer [11-13]. Antibiotics, ARB, and ARGs can enter surface water through diverse anthropogenic pathways [14]. Previous studies have reported elevated levels of ARB and ARGs in municipal WWTP effluent, confirming that treated wastewater discharges significantly contribute to the presence of antibiotics and antibiotic resistance in the aquatic environment [15-19]. For example, tetracycline and sulfonamide were reported in WWTP effluent, at mean total concentrations of 652.6 and 261.1 ng/L, along with mean concentrations of corresponding resistant bacteria of 1.05×10^1 and 3.09×10^3 CFU/mL, respectively [15]. A significantly higher ciprofloxacin resistance percentage was observed in treated effluent compared with the respective raw influent [16, 17]. Resistances were found against ciprofloxacin (14%), erythromycin (24.8%), and tetracycline (34.6%) for isolates investigated in 42 samples of influent, treated effluent, and sludge from 14 municipal WWTPs [18]. Impacts from discharged antibiotics were observed significantly downstream of the discharge point. For example, Sabri et. al (2018) detected several antibiotics and their corresponding ARGs down to 20 km from WWTP discharge point within stream surface water and sediment samples [19].

A recent study highlights the national occurrence of ARGs in U.S. waters: class 1 integron interphase (i*ntI1*), sulfonamide resistance (*sul1*), and tetracycline (*tetw*) genes were present in 54%, 49%, and 34% of the 1,800 samples, respectively [20]. Other prior studies have analyzed ARG occurrence by focusing on linkages among the occurrence of a limited number of antibiotics and selected ARGs in environmental settings [21-23]. However, in doing so previous studies have been performed on a site-by-site basis, with site selection largely based on local knowledge of the area and limited in sample size by cost and time. Supporting in-stream analysis with model estimates offers a useful supplement to the costly and timely nature of obtaining large sample sizes. Model estimates for in-stream concentrations have been limited by a lack of streamflow data for low-flow conditions, until a recent deployment of low instream flow estimates for all USGS NHDPlus flowlines by iSTREEM V2.2 [24].

In the U.S. and most other countries such as Canada [25], China [26], and Germany [27], regulatory systems for pharmaceutical residues have not considered environmental antibiotic concentrations that may expedite the development and spread of resistant pathogens [28, 29]. This is in part due to fragmented information indicating the extent of antibiotic contributions to the ARB development in the enrvironment[2], further challenging quantification of antibiotic concentrations that take such risks into account [30]. Although databases of clinical breakpoints established by European agencies (ECDC, EUCAST) [31, 32] have provided some standardization and organization of antibiotic resistance data, use for the implementation of antibiotic emission limits into the external environment is not reliable. In light of this challenge, Bengtsson-Palme and Larsson modeled predicted no-effect concentrations (PNECs) for selection of resistance for common antibiotics, which in most cases were lower than previously reported PNECs considering ecotoxicological effects[28]. In addition, there are experimental studies assessing selective concentrations of antibiotics using complex microbial communities. For example, selective concentrations in complex aquatic bacteria biofilm have been determined for ciprofloxacin (100 ng/L)[33] and tetracycline (≤ 100 g/L)[34].

In this study, we used Bengtsson-Palme and Larsson's [28] and Kraupner et al's [33] PNEC approach for antibiotic resistance as a benchmark to identify streams more susceptible to antibiotic concentrations by comparing the PNEC to model estimates of ciprofloxacin (CIP), erythromycin (ERY) and sulfamethoxazole (SMX) within WWTP effluent receiving systems across the U.S. The previously developed De Facto Reuse

Incidence Nations Consumable Supply (DRINCS) model [35, 36] is expanded here to estimate antibiotic concentrations during low instream flow events across the U.S., overcoming the previous limitation of stream gauges being present on roughly 8% of the discharge sites. This allows for model estimates of ciprofloxacin, erythromycin, and sulfamethoxazole in streams receiving WWTP discharges during average and low instream flow conditions. Using these estimates, we examine the potential for ciprofloxacin, erythromycin, and sulfamethoxazole resistance by comparing these estimates to the PNECs with a standard safety factor applied. Results are then geospatially compared across the U.S. to identify hydrologic regions more prone to ciprofloxacin, erythromycin, and sulfamethoxazole resistance.

3.3 MATERIALS AND METHODS

3.3.1 PREDICTING ANTIBIOTIC CONCENTRATIONS USING DRINCS

The previously developed DRINCS model was updated with the most current Clean Watershed Needs Survey (CWNS) data and expanded to include low instream flow estimates for the majority of streams across the U.S. DRINCS is an ArcGIS model that couples python script with spatial datasets to create a network of flowline and conduct mass balances on wastewater discharged to surface streams[35, 36]. Complete model details and validation can be found in our prior study[35]. WWTP locations have been updated for CWNS 2012 [37] and hydrography data obtained USGS National Hydrography Data Set Plus Version 2 (NHDPlus) [38]. DRINCS was also updated to expand low instream flow estimates to contain 7-day 10-year low-flow (7Q10), which was obtained from iSTREEM V2.2 [24]. Out of 13,245 WWTPs with surface water discharge, 389 (nearly 3%) did not have estimates for 7Q10. Dilution factors (DFs) for each receiving stream were calculated according to Equation 1, where Q_w is WWTPs present design flow and Q_r is the streamflow of the receiving stream under average or low instream flow conditions.

$$DF = \frac{Q_r + Q_w}{Q_w} \tag{1}$$

Antibiotic concentrations were calculated at discharge sites by dividing the median of the measured environmental concentrations (MECs) to predicted dilution factors. Kelly and Brooks (2018) and Schafhauser et al. (2018) investigated the global occurrence of ciprofloxacin and erythromycin, respectively, in several different water matrixes including municipal WWTPs effluent[39, 40]. Available data for ciprofloxacin, erythromycin, and sulfamethoxazole indicated 193.2, 107, and 385 ng/L as the median of MECs in WWTPs effluent, whereas the 90th percentile of MECs were 8,745, 331.5, and 1,418 ng/L, respectively [39, 40] (*Appendix*, Table A1).

3.3.2 IDENTIFYING STREAMS MORE SUSCEPTIBLE TO ANTIBIOTIC RESISTANCE

Hazard quotients were calculated for ciprofloxacin, erythromycin, and sulfamethoxazole within effluent by Equation (2) where MECs for each antibiotic were taken from the literature [40, 41] and estimated environmental concentrations (EECs) were modeled as the median and 90th percentile reported values. PNECs for resistance selection for each antibiotic estimated by Bengtsson-Palme and Larsson or Kraupner et al were set as an endpoint that is predicted to be protective against the promotion of resistance [28,49]. The dilution factor required to meet the hazard quotient for each antibiotic was estimated using Equation (3), where HQ_{eff} is the hazard quotient for each antibiotic in the municipal effluent, and LOC is the level of concern considering a recommended safety factor of 10 (0.1)[42].

$$HQ_{eff} = \frac{EEC}{PNEC} \tag{2}$$

$$DF_{req} = \frac{HQ_{eff}}{LOC} \tag{3}$$

Bengtsson-Palme and Larsson (2016) estimated PNECs for resistance selection for erythromycin and sulfamethoxazole equal to 1000 and 16,000 ng/L[28]. Also, Kraupner et al. (2018) determined a selective concentration for ciprofloxacin to be 100 ng/L. Therefore, we calculated the dilution factor required to meet the antibiotic resistance PNEC with a ten-fold safety factor for ciprofloxacin, erythromycin, and sulfamethoxazole equaling to 19.32, 1.07, and 0.24, where the EECs are modeled as the median of the MECs for each antibiotic (*Appendix,* Table A2). The resulting value is referred to as the ABR safety threshold throughout the following text. Discharge sites with higher modeled antibiotic concentrations or lower dilution factors were considered to be prone to antibiotic resistance. A complete discussion of the model performance and limitations can be found in the *SI Appendix,* Study Limitations, and Model Performance.

3.4 RESULTS AND DISCUSSION

3.4.1 ANTIBIOTIC OCCURRENCE AND PNEC EXCEEDANCE UNDER MEAN ANNUAL INSTREAM FLOW

We calculated dilution factor and antibiotic concentrations at the discharge sites of 13,245 WWTPs under mean annual instream flow. All WWTP discharge sites are depicted in Figure 1 and color-coded based on the dilution factor for each receiving stream. Figure 1a shows the spatially distributed dilution factor under mean annual instream flow, where the 25th, 50th and 75th percentiles of dilution factors are 9.7, 55.4, and 422.8, respectively, which are higher than the previously reported statistics of 8, 43, and 287 for 25th, 50th and 75th percentiles based on CWNS 2008 data[43]. Dilution factor plays a significant role in the presence and magnitude of antibiotics present in surface water. Because effective exposure duration is elevated in effluent-dominated and dependent systems [44], the lower dilution factor is associated with higher antibiotic concentrations and higher potential occurrence of antibiotic resistance in surface water. Our results indicate the 25th, 50th and 75th percentiles of calculated ciprofloxacin concentrations within receiving streams are 0.46, 3.48, and 20.01 ng/L respectively, where the estimated environmental concentrations (EECs) are modeled as the median of the reported value. Consequently, more than one-third (*34.9%, n=4,629*) of sites surpassed the antibiotic resistance (ABR) safety threshold for ciprofloxacin under mean annual instream flow. Alternatively, the 25th, 50th, and 75th percentiles of erythromycin concentrations are 0.25, 1.93, and 11.08 ng/L, and only 1.2 % of the sites (n=154) exceeded this ABR safety threshold. In contrast, sulfamethoxazole ABR exceedance is negligible for average and low instream flow conditions, due to low sulfamethoxazole concentrations (6.94 ng/L [50th percentile]) paired with a high PNEC (16,000 ng/L).

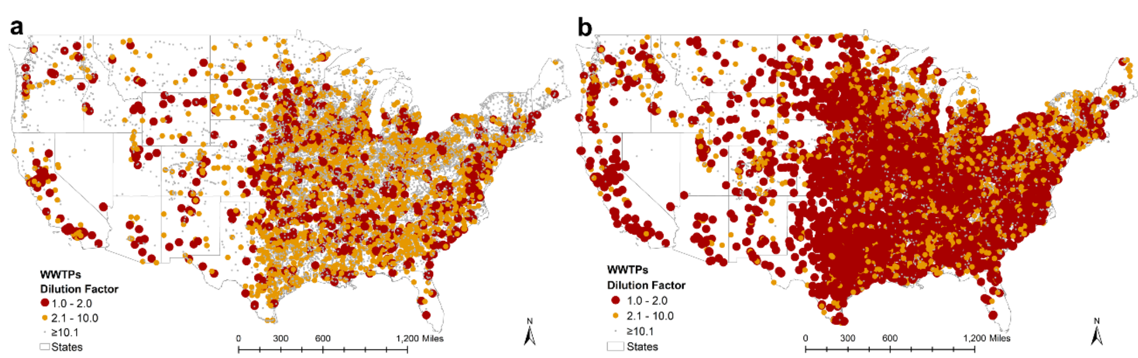

Figure 1. Color-coded maps of dilution factors for receiving streams at the wastewater treatment plant (WWTP) discharge sites under (A) mean instream flow conditions and (B) 7-day, 10-year low instream flow (7Q10) conditions across the U.S.

Figure 2a displays the geospatial distribution of discharge sites that exceeded the ABR safety threshold for ciprofloxacin and erythromycin under mean annual streamflow. The map is normalized based on the total number of WWTPs within the 4-digit USGS watershed boundary condition (HUC-4). As a result, 11 out of 202 HUC-4 watersheds exceeded ciprofloxacin ABR safety thresholds for greater than 80% of WWTP discharge sites. These watersheds are located across Arizona, California, Florida, Georgia, Nevada, New Mexico, Texas, and Utah. In addition, 50% of discharge sites did not meet the ciprofloxacin ABR safety threshold in 46 out of 202 HUC-4 watersheds. It is therefore important to note that the aforementioned data is modeled based on mean-flow conditions and the median of the MECs for ciprofloxacin; hence, we expect to see a higher magnitude of antibiotic concentration and a more severe occurrence of antibiotic resistance under low instream flow conditions due to insufficient dilution factors.

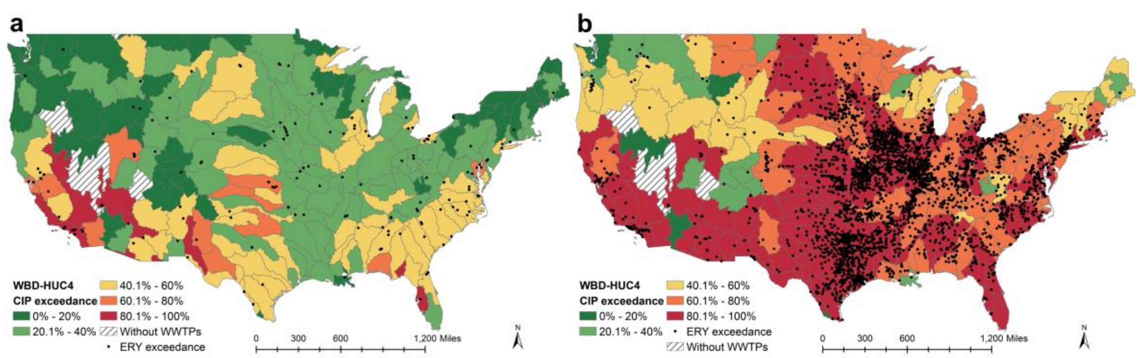

Figure 2. Map of the percentage of sites exceeding antibiotic resistance (ABR) thresholds for ciprofloxacin (CIP; color-coded) and erythromycin (ERY; dots) based on four-digit hydrologic units (A) under mean instream flow conditions, and (B) 7Q10 (7-day, 10-year) low instream flow conditions. EECs are modeled as the median literature MECs value. The percentage of ciprofloxacin exceedance is equal to the number of sites that do not meet the safety threshold for ciprofloxacin divided by the total number of wastewater treatment plants (WWTPs) within each hydrologic unit (HUC-4). The sites that do not meet the AMR safety threshold for erythromycin are shown as dots.

3.4.2 EFFECTS OF LOW INSTREAM FLOW CONDITIONS ON ANTIBIOTIC CONCENTRATIONS AND PNEC EXCEEDANCE

Streamflow is a key factor in antibiotic concentrations and potential antibiotic resistance presence in streams affected by WWTPs. We investigated the influences of instream flow conditions on dilution factors for each WWTP discharge site. Dilution factors may decrease during seasonal low-flow or drought conditions; therefore, the ABR safety threshold for a particular antibiotic is more likely to be exceeded due to insufficient dilution because effective exposure duration is elevated [44]. Dilution factor and antibiotic concentrations were calculated at the discharge sites of 12,856 WWTPs with available low instream flow data (97.1% of all sites). Under low instream flow conditions modeled as 7-day 10-year low-flow (7Q10), 48.0% ($n= 6,175$) and 70.4% ($n=9,052$) of WWTPs have a dilution factor below 2 and 10 respectively, in contrast to only 6.2% ($n=826$) and 25.4% ($n=3,368$) under average instream flow conditions, confirming the significant influences of streamflow on dilution of reclaimed wastewater. Compared to average-flow conditions, median dilution factors decreased from 55.4 to 2.4 under low-flow conditions. In this case, the 25th and 75th percentiles of dilution factors were 1.1 and 20.0, respectively. Our findings indicate the 25th, 50th, and 75th of antibiotic concentrations in the U.S. receiving streams were 9.66, 80.77, and 175.79 ng/L for ciprofloxacin; 5.35, 44.73 and 97.36 ng/L for erythromycin; and 19.26, 160.95 and 350.30 ng/L for sulfamethoxazole.

Under low instream flow conditions, dilution factors in 76.9% ($n=9,885$) of streams did not meet the ABR safety factor for at least one antibiotic compound examined here (primarily ciprofloxacin). Additionally, 25.8% ($n=3,323$) did not meet the

ABR safety threshold for two antibiotics under low instream flow conditions (ciprofloxacin and erythromycin). Figure 2b shows the geospatial distribution of discharge sites that exceeded the ABR safety threshold for ciprofloxacin and erythromycin under mean low instream flow conditions. Consequently, 98 of 202 HUC-4 watersheds exceeded ciprofloxacin safety thresholds for greater than 80% of WWTPs within their respective hydrologic regions. Furthermore, 50% of discharge sites did not meet the ciprofloxacin ABR safety threshold in 169 out of 202 HUC-4 watersheds. These sites can be considered as potential sites of antibiotic resistance caused by low instream flow conditions. Our findings indicate that under low-flow stream conditions, antibiotic concentrations can increase by up to four magnitudes due to insufficient stream flow, which can be exacerbated by droughts and water extraction, resulting in higher PNEC exceedance for antibiotic resistance.

3.4.3 IMPACT OF MONTHLY INSTREAM FLOW VARIATIONS ON ANTIBIOTIC CONCENTRATIONS AND POTENTIAL ANTIBIOTIC RESISTANCE PRESENCE

Wastewater contributions can vary greatly not only by low instream flow events (i.e., 7Q10) but also temporally by monthly streamflow variations [45]. We calculated antibiotic concentrations and corresponding ABR safety threshold exceedances based on the mean monthly instream flow for all sites obtained from NHDPlus V2.0 (*n=13,245* discharge sites). Overall, monthly streamflow for the majority of modeled discharge sites is highest in March and lowest in August; therefore, we have set March and August as periods of high and low seasonal instream flow for comparison (as shown in *Appendix,* Figure A3). When compared to mean annual flow, the median dilution factor across sites in March shows an increase of 191%, whereas, in August, the median dilution factor decreased by 62%. Under low seasonal flow conditions in August, 13.6% *(n= 1,796)* and 39.5% *(n=5,230)* of WWTPs had dilution factors below 2 and 10 respectively, in contrast to only 3.5% (*n=460*) and 14.4% (*n=1,912*) in March. Under high seasonal flow conditions in March, the 25th, 50th, and 75th percentiles of calculated ciprofloxacin concentrations within U.S. streams receiving wastewater effluent are 0.20, 1.20, and 7.28 ng/L, respectively, where the EECs are modeled as the median of the reported value. Consequently, 21.1% (*n=2,794*) of sites surpassed the antibiotic resistance (ABR) safety threshold for ciprofloxacin in March, which is a decrease of 1,835 sites when compared to mean annual streamflow conditions. Alternatively, the 25th, 50th, and 75th percentiles of erythromycin concentrations are 0.11, 0.66, and 4.03 ng/L, and only 1.1 % of the sites (*n=149*) exceeded the ABR safety threshold. The median of sulfamethoxazole concentrations under high monthly flow conditions is 2.39 ng/L. However, ABR exceedance is negligible for high and low monthly streamflow conditions, due to the high PNEC (16,000 ng/L) of sulfamethoxazole.

Compared to March conditions with higher flows, median dilution factors decreased from 161.0 to 21.2 in August, where the 25th and 75th percentiles of dilution factors were 4.0 and 176.3, respectively. Our results showed that the 25th, 50th, and 75th percentiles of antibiotic concentrations at discharge sites were 1.10, 9.12, and 48.67 ng/L for ciprofloxacin; 0.61, 5.05, and 26.96 ng/L for erythromycin; and 2.18, 18.18 and 97.00 ng/L for sulfamethoxazole. Consequently, 48.8% (*n=6,465*) streams did not meet the ABR safety factor for ciprofloxacin, which is an increase of 1836 sites when compared to the mean annual streamflow. *Appendix,* Table A3 shows the number of sites that exceeded the ciprofloxacin threshold by month and Strahler stream order (described in more detail in the following section), where exceedance is increased in smaller streams (SSO 1 and 2) for July through October. In addition to ciprofloxacin exceedance, 2.7% *(n=364)* of the sites exceeded the ABR safety threshold for erythromycin. Our findings indicate the importance of seasonal variations in streamflow on estimated antibiotic instream concentrations and the likelihood for ABR PNEC exceedance.

3.4.4 THE ROLE OF STRAHLER STREAM ORDER IN ANTIBIOTIC RESISTANCE PNEC EXCEEDANCE

Strahler stream order (SSO) uses a hierarchy of tributaries to define stream size and has proven to play an important role in the impact of discharging WWTPs to receiving streams[43]. Climate-related streamflow variation is expected to have bigger impacts on receiving streams with lower Strahler stream order (i.e., smaller-sized streams)[35]. In addition, smaller-order streams often rely on wastewater discharges as a sustainable source of instream flow streams[45]. Consequently, effluent receiving streams with smaller Strahler stream orders are more susceptible to higher antibiotic concentrations and exposure. Approximately 64% (*n=8,459*) of all WWTPs discharge to streams classified by a Strahler stream order of 3 or lower (*Appendix*, Figure A2), resulting in a median dilution factor of 18.6 and 1.3 under mean annual flow conditions and low instream flow conditions respectively. *Appendix*, Figure A3 shows antibiotic concentrations at discharge site grouped by Strahler stream order under mean annual and low instream flow conditions. According to *Appendix*, Figure A3, streams with lower Strahler stream order experience higher antibiotic concentrations due to lower stream potential to buffer antibiotic loadings which may lead to increased antibiotic resistance presence. The median of antibiotic concentrations for ciprofloxacin, erythromycin, and sulfamethoxazole at discharge sites classified by a Strahler stream order equal or lower than 3 are 10.39 ng/L, 5.75 ng/L, and 20.70 ng/L under mean annual flow conditions, compared to 3.48 ng/L, 1.93 ng/L, and 6.94 ng/L for all SSO categories combined.

Figures 3a and b display dilution factors categorized by Strahler stream order and the dilution factor required for each antibiotic to fall below the ABR PNEC hazard quotient given a ten-fold factor of safety. Figure 3c shows U.S. major streams color-coded according to Strahler stream order. Generally, sites along a stream equal to or less than the Strahler stream order of 5 are more prone to exceeding an ABR safety threshold for at least one antibiotic compound. Under mean annual streamflow, a total number of 4,629 out of 13,245 sites exceeded the ABR safety threshold for ciprofloxacin where 92.7% *(n=4,292)* of them discharge effluent to streams with Strahler stream order of equal to or less than 3. Figure 3b shows that almost all streams characterized by Strahler stream order less than or equal to 3 exceeded the ciprofloxacin ABR safety threshold under low instream flow conditions (modeled as 7Q10). Our results indicate that PNEC exceedance is exacerbated for sites located on lower SSO streams (SSO$\leq$ 4), which represents 77.1% of all WWTP discharges.

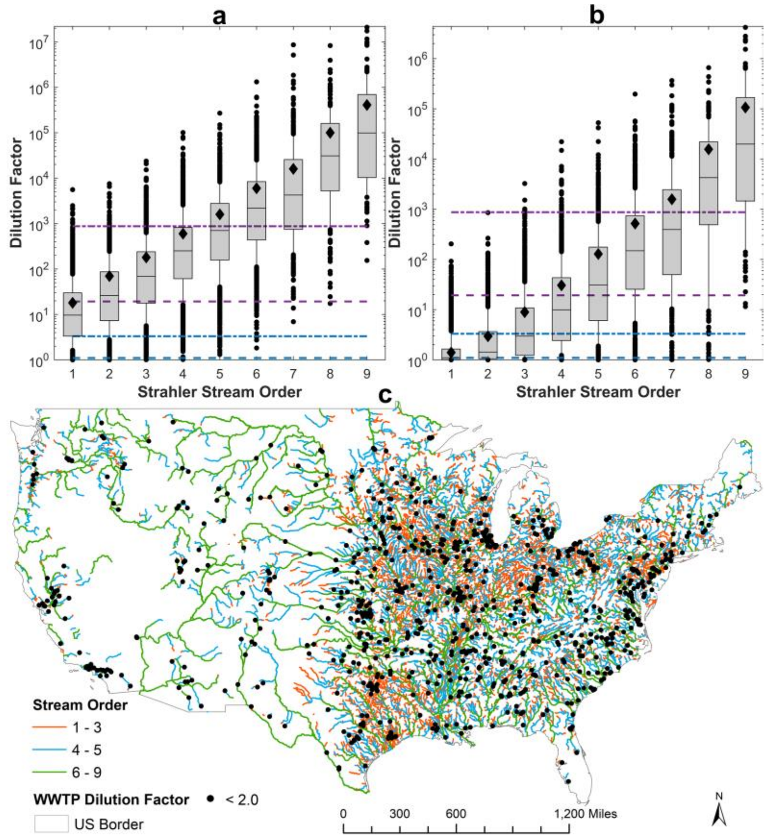

Figure 3. Dilution Factor *DF) versus Strahler stream order across the U.S. (A) under mean instream flow conditions. (B) under 7Q10 (7-day, 10-year) low instream flow conditions. Purple lines indicate dilution factor required for ciprofloxacin and blue lines represent dilution factor for erythromycin to fall below a hazard quotient for antibiotic resistance considering a ten-fold safety factor, where '--' represent EECs modeled as the median of MECs value and '-.' represent EECs modeled as the 90th percentile of the MECs value. Top and bottom of the box: 75th and 25th percentiles, respectively; top and bottom of whisker:90th and 10th percentiles, respectively; line inside the box:50th percentile (median); diamond (♦): average. (C) Color-coded map for Strahler stream order for major streams across the U.S. WWTPs with dilution factor below 2 at discharge sites are represented by dots (•).

3.4.5 INFLUENCE OF KEY PARAMETER ESTIMATION ON PNEC EXCEEDANCE

Here we investigated the sensitivity of our modeled PNEC exceedance to changes in instream flow, WWTP effluent flow rates, estimated environmental concentrations, and the applied safety factor. Empirical cumulative distribution functions were developed for ciprofloxacin concentrations based on each modeled scenario and compared against the PNEC threshold set with varying levels of concern (i.e., safety factors of 1, 0.05, and 0.1). Ciprofloxacin was selected due to having the highest PNEC exceedance in respect to the antibiotics investigated in this study. To investigate the role of instream flow condition on PNEC exceedance, streamflow values were set to 7Q10, mean annual, highest, and lowest monthly mean indices for instream flow. Figure 4a shows the percentage of sites with CIP concentrations that exceed PNEC thresholds for varying factors of safety. With a tenfold safety factor, more than 76% of the dataset exceeds the PNEC threshold when instream flow is modeled by 7Q10; contrastingly only 21.1% of sites exceeded ABR PNEC during the highest mean monthly streamflow conditions. The influence of streamflow is very pronounced, particularly for low instream flow indices. It's important to note that while 7Q10 has a ten-year frequency, the lowest mean monthly is a seasonal estimate under which 48.8% of sites are estimated to be above the ABR PNEC when a tenfold safety factor is applied. In a similar manner, we investigated the impact of effluent flow on PNEC exceedance by setting effluent flow equal to present design flow, 50% and 75% of present design flow; to align with reported flow data for WWTP discharges [46, 47]. Figure 4b shows that impacts to PNEC exceedance are less profound for effluent variations than alterations to streamflow. PNEC exceedance is more pronounced in smaller streams when EEC is modeled as 90th percentile of MEC, instead of median MEC (see *Appendix,* Figure A4 and Table A4). The total number of sites that did not meet the dilution factor required for antibiotic resistance decreased by 1,280 and 575 when the effluent flow is estimated at 50% and 75% of the present design flow, compared to the present design flow (*N = 4,629*). The figures provided in this section allow for the estimation of PNEC exceedance based on multiple scenarios.

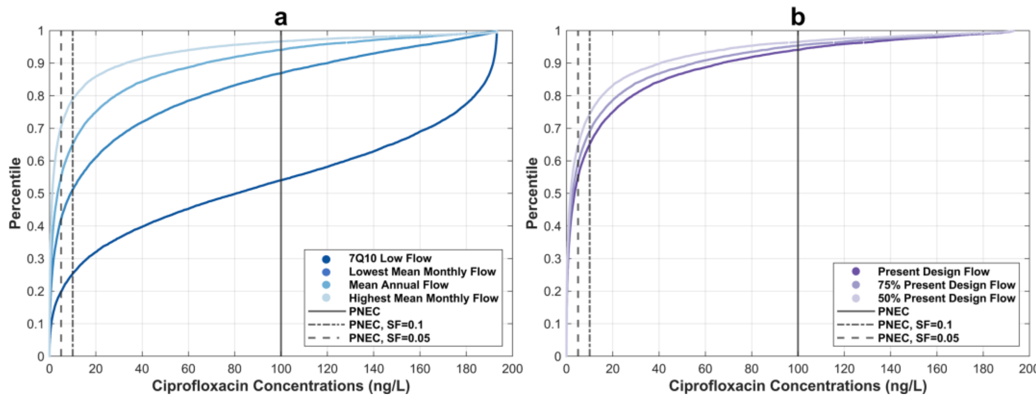

Figure 4. The empirical cumulative distribution functions of ciprofloxacin concentrations (ng/L) under (A) varying instream flow conditions including 7Q10 (7-day, 10-year) low flow, lowest mean monthly flow (August), mean annual flow, and highest mean monthly flow (March), (B) varying effluent flow conditions including present design flow, high WW flow (75% of present design flow), and medium WW flow (50% of present design flow).

3.5 CONCLUSION

In this study, the likelihood of an antibiotic presenting a resistance threat within a specific watershed is largely dependent on its concentration within wastewater effluent and the PNEC for selective pressure. Ciprofloxacin, classified as an essential medicine by the World Health Organization, is a case where a low ABR PNEC (100 ng/L) and relatively high concentrations in treated effluent (median MEC= 193.2 ng/L) yields antibiotic resistance concerns for numerous sites during average and low instream flow conditions across the U.S. Despite the extensive therapeutic use and resulting concentrations of erythromycin and sulfamethoxazole in treated effluent (median MEC = 107 and 385 ng/L), the threat of antibiotic resistance is relatively lower due to a higher ABR PNEC (1,000 and 16,000 ng/l). We expect this trend to hold true for other antibiotic compounds with similar characteristics. Additionally, prior work suggests combinations of different antibiotics within wastewater could substantially increase respective antibiotic resistance [48-50]. Future work investigating the combined effects of antibiotic resistance will allow for a more complete investigation of the role of WWTPs in antibiotic resistance occurrence within the aquatic environment. The implementation of the DRINCs model in identifying the most impacted streams by antibiotic resistance is limited by the lack of knowledge on antibiotics and antibiotic resistance fate and transport in the aquatic environment and the parameter values used in the model, including instream flow, antibiotic EECs, effluent flow rate and the factor of safety. The outcome of the parameter analysis done to address some of these limitations indicates that model prediction is more impacted by instream flow conditions, safety factor, and antibiotic EECs than effluent flow. Despite these limitations, DRINCS is a powerful tool in identifying the spatial distribution of streams most likely to be impacted by antibiotic resistance and comparing relative differences among streams rather than precisely quantifying antibiotic and resistance gene concentrations. Integrating DRINCS model estimates into future field studies will help to identify locations where antibiotic resistance is more likely to be present within lotic ecosystems and such information can be used to prioritize antibiotic and ABR monitoring and potential interventions across the U.S.

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3.7 APPENDICES

3.7.1 STUDY LIMITATIONS AND MODEL PERFORMANCE

This study primarily focused on identifying receiving streams most impacted with antibiotics from wastewater treatment plant (WWTP) discharges and predicting the geospatial distribution of sites with antibiotic concentrations at levels of concern for antimicrobial resistance. Several assumptions are made to complete a study of this nature at the national scale, which leads to limitations on how the results should be interpreted. In completing the study, we focus on antibiotic residues and do not account for antibiotic resistant genes or bacteria (ARGs and ARB) directly discharged from WWTPs into the surface water. However, sites expected to have relatively higher loadings of ARGs and ARB from WWTPs are indirectly identified because they correspond to sites that are predicted to have higher antibiotic concentrations relating to higher ratios of effluent to instream flow. We limit our evaluation of antibiotic loading to WWTP sources, however additional anthropogenic sources and land use activities are positively correlated with antibiotic resistance209. Although our model did not consider the exposure duration to antibiotics required for the development of resistance at the discharge sites, we assumed continuous discharge of effluent containing antibiotic and antibiotic resistance to the surface water may lead to more antibiotic resistance susceptibility at the vicinity of discharge sites than further downstream in the river network.

In this study, we investigated three different antibiotics including ciprofloxacin, erythromycin, and sulfamethoxazole. Ciprofloxacin is a DNA gyrase inhibitor preventing cell replication[209], erythromycin prevents peptide synthesis by targeting 70S RNA ribosome peptide synthesis[210], and sulfamethoxazole targeting synthesizing of tetrahydrofolic acid by hindering an enzyme leads to alternation of thymidine metabolic pathway[211]. Despite the differences in use, kinetics, and mechanism of action, ciprofloxacin, erythromycin, and sulfamethoxazole have been documented as contaminants of emerging concern[212-216], especially due to extensive consumption rate, high persistency, and potential toxicity in the aquatic environment[192].

In addition, different types of treatment technologies employed by WWTPs can decrease antibiotic and antibiotic resistance bacteria concentrations and the enrichment of ARGs in the treated effluent to different degrees. However, the effect of the treatment level is not involved in the model in depth. Although the model uses one estimate for selected antibiotic concentrations within effluent across all WWTP without considering treatment level, the majority of WWTPs with surface water discharge across the U.S. implemented secondary treatment (n=7861 out of 13245 WWTPs), which were not designed to remove antibiotics and antibiotic resistance. Environmental estimated concentrations (EECs) of antibiotics used in the model are calculated from reported values of WWTPs with primary, secondary, and tertiary treatment levels. We addressed the uncertainty of concentrations within wastewater effluent by using different statistics including the 90th percentile and the median of reported values. Furthermore, the effluent flow data used in the model including present design flow and existing flow from the CWNS dataset provide an overestimate of the actual effluent flow of WWTP. In addition, the degradation of antibiotics in the natural environment is not included in the model because antibiotics selected for this study are relatively persistent in the aquatic environment and degradation mechanisms are not well understood. To understand the role of our assumptions on the estimated levels of antibiotic resistance PNEC exceedance we varied key model parameters including instream flow conditions (i.e., 7Q10 low flow, lowest, mean monthly flow, mean annual flow, and highest mean monthly flow), effluent flow (i.e., present design flow, 75 and 50% of present design flow), ciprofloxacin EECs (i.e., median and 90th percentile and of MECs) and level of concern (i.e., safety factors of 1, 0.05 and 0.1). Results of this analysis are presented in the "Influence of key parameter estimation on PNEC exceedance" section in the article's main text.

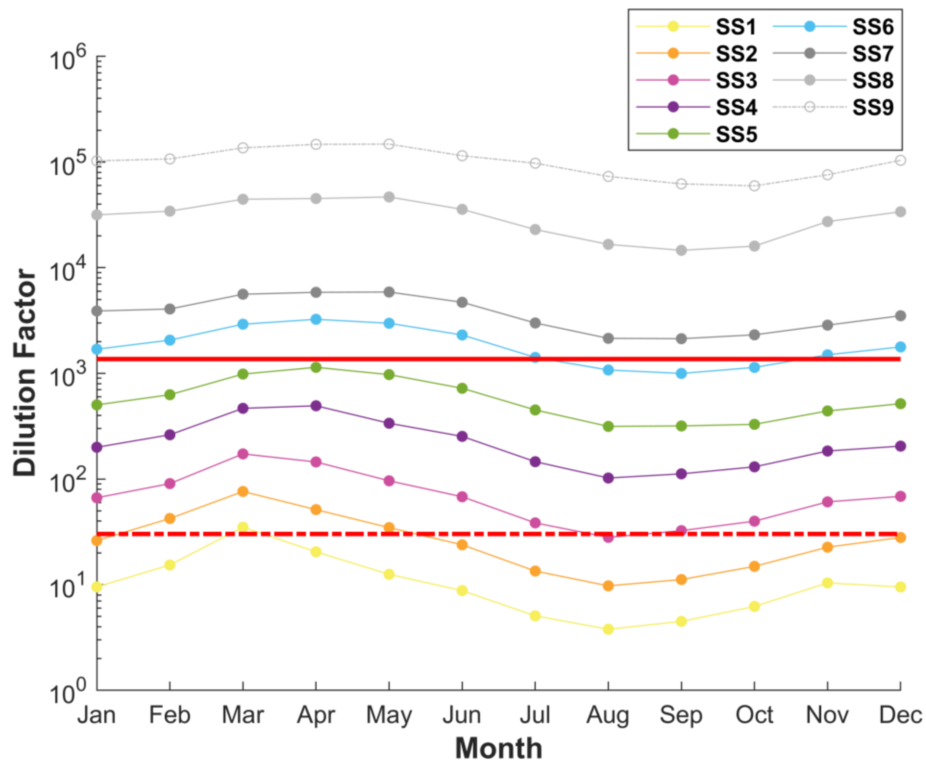

Figure A1. Median of dilution factors (DF) across all WWTPs under temporal variation (modeled by monthly streamflow conditions) and grouped by Strahler Stream Order. Red Lines represent the DF required for ciprofloxacin to fall below a hazard quotient for antibiotic resistance given a ten-fold safety factor, where '-' represents EEC modeled as 90th percentile of MECs and '-.' represents EEC modeled as the median of MEC.

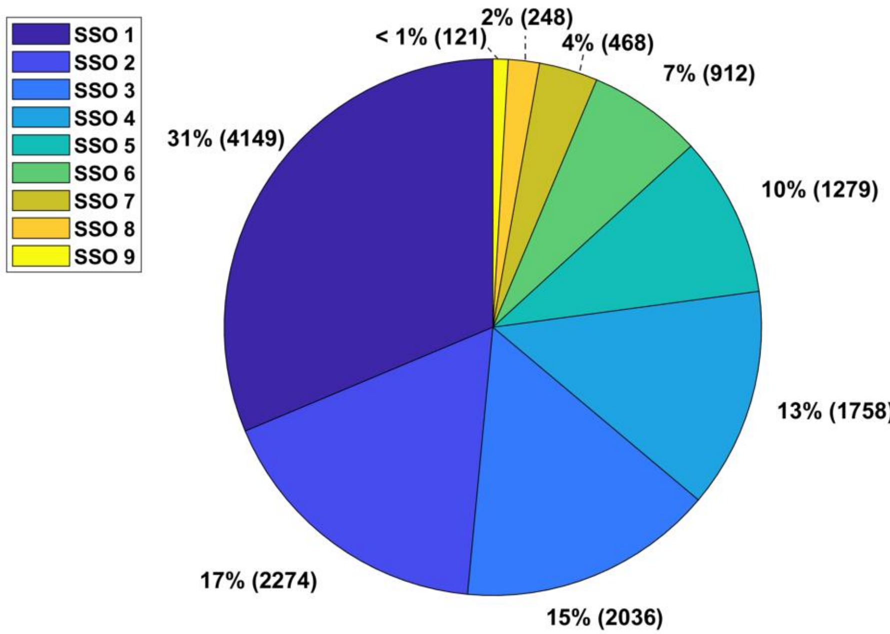

Figure A2. Number of WWTPs with discharge to surface water across the U.S. categorized by Strahler Stream Order (SSO).

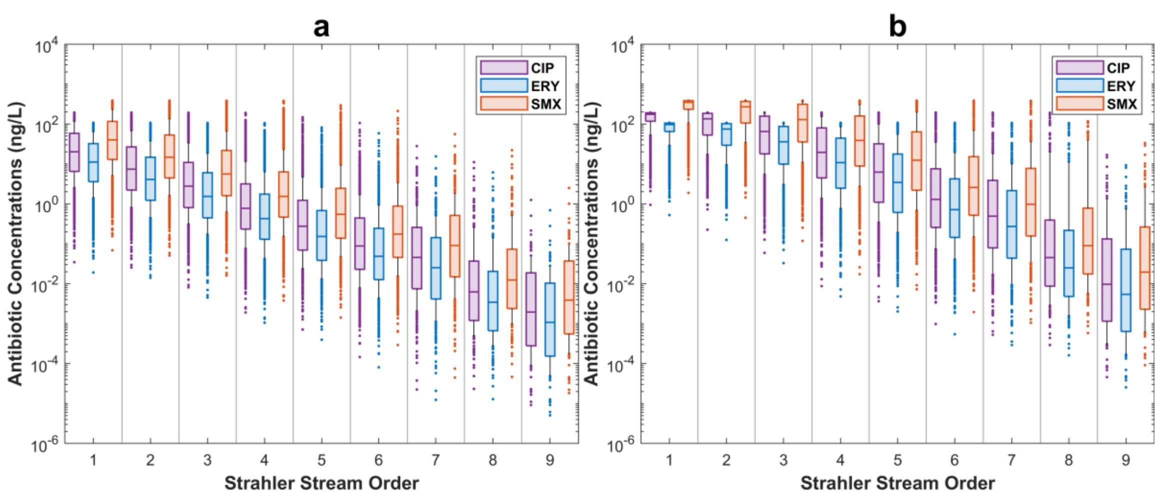

Figure A3. Antibiotic concentrations (ng/L) at discharge sites grouped by Strahler Stream Order under a) mean annual flow conditions and b) 7Q10 (7-day, 10-year) low instream flow conditions.

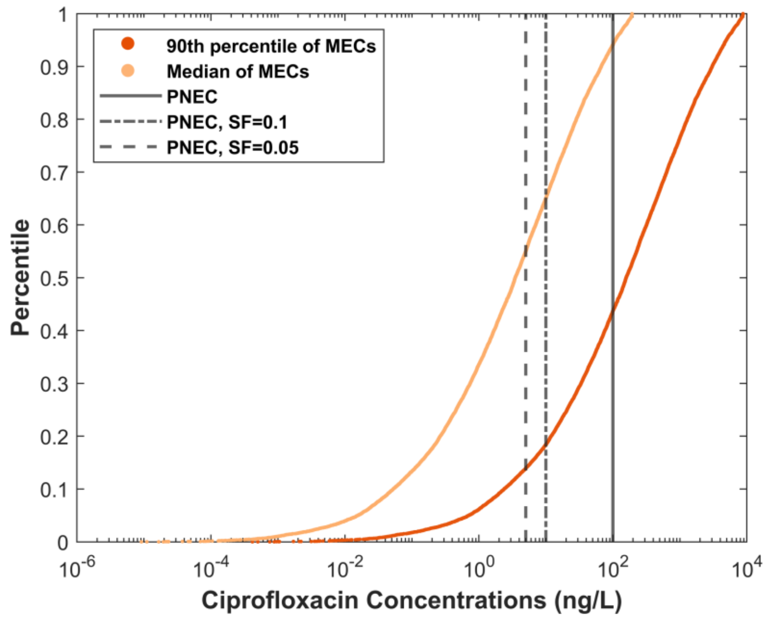

Figure A4. The empirical cumulative distribution functions of ciprofloxacin concentrations (ng/L) when EEC is modeled as 90th percentile of MECs and the median of MECs.

Table A1. Literary values for measured environmental concentrations of sulfamethoxazole in treated wastewater effluent.

Reference	Location	Country	WWTP name	Matrix	Effluent (ng/L)			
					Min	Max	Mean	Median
Barber et al. (2013)[217]	Boulder Creek, Colorado	USA	the City of Boulder's WWTP	Secondary Effluent			160	
	Fourmile Creek, Iowa	USA	the City of Ankeny's WWTP	Secondary Effluent			720	
Kwon & Rodriguez (2014)[218]	Jackson, Mississippi	USA	Savanna WWTP & 2 more	Secondary Effluent		1328	304	61
Batt et al. (2006)[132]	Erie County, New York	USA	East Aurora WWTP	Secondary Effluent			370	
	Erie County, New York	USA	East Aurora WWTP	Secondary Effluent			410	
	Erie County, New York	USA	Holland WWTP	Secondary Effluent			600	
	Erie County, New York	USA	East Aurora WWTP	Secondary Effluent			660	
	Erie County, New York	USA	Lackawanna WWTP	Secondary Effluent			700	
	Erie County, New York	USA	Lackawanna WWTP	Secondary Effluent			1100	
	Erie County, New York	USA	Holland WWTP	Secondary Effluent			1300	
	Erie County, New York	USA	Holland WWTP	Secondary Effluent			1340	
Batt et al. (2007)[44]	Erie County, New York	USA	East Aurora WWTP	Secondary Effluent			220	
	Erie County, New York	USA	Lackawanna WWTP	Secondary Effluent			380	
	Erie County, New York	USA	Holland WWTP	Secondary Effluent			500	
	Erie County, New York	USA	Amherst WWTP	Secondary Effluent			680	
Blair et al. (2013)[219]	Milwaukee, Wisconsin	USA	Jones Island WRF	Tertiary Effluent	7.7	77	29	
	Milwaukee, Wisconsin	USA	South Shore WRF	Tertiary Effluent	0	14	6.9	

Bradley et al. (2014)[220]	Ankeny, Ohio	USA	Ankeny WWTF	Secondary Effluent			900	
	Ankeny, Ohio	USA	Ankeny WWTF	Secondary Effluent			1650	
	Ankeny, Ohio	USA	Ankeny WWTF	Secondary Effluent			2210	
Gibs et al. (2013)[221]	New Jersey	USA	Northwest Bergen County WWTP	Secondary Effluent			54	
	New Jersey	USA	Ridgewood WWTP	Secondary Effluent			153	
Karpuzcu et al. (2014)[222]	Minnesota	USA	South Fork of the Zumbro River WWTP	Final Effluent	1100	2000	1600	
	Minnesota	USA	South Fork of the Zumbro River WWTP	Final Effluent	50	750	480	
	Minnesota	USA	South Fork of the Zumbro River WWTP	Final Effluent	250	550	480	
Karthikeyan (2006)[223]	Wisconsin	USA	-	Final Effluent			270	
Mitchell & Ullman (2016)[224]	Spokane, Washington	USA	Spokane WWTP	Secondary Effluent			901	
Nelson et al. (2010)[225]	Los Angeles, California	USA	-	Tertiary Effluent			18	
	Los Angeles, California	USA	-	Tertiary Effluent			180	
	Los Angeles, California	USA	-	Tertiary Effluent			265	
	California	USA	-	Tertiary Effluent			18	
	California	USA	-	Tertiary Effluent			180	
	California	USA	-	Tertiary Effluent			265	

Parry & Young (2016)[226]	Davis, California	USA	UC Davis WWTP	Tertiary Effluent			110	
Renew et al. (2004)[227]	Georgia, California, and Arizona	USA	-	Secondary Effluent		640	5575	
	Georgia, California, and Arizona	USA	-	Secondary Effluent	100	1600	395	
Spongber et al. (2008)[228]	Ohio	USA	-	Final Effluent			79.4	
	Ohio	USA	-	Final Effluent			273.7	
	Ohio	USA	-	Final Effluent			472.4	
Vanderford & Synder (2006)[229]	Las Vegas, NV	USA	The City of Las Vegas Water Pollution Control Facility	Tertiary Effluent			24	
Wert et al. (2009)[230]	Colorado	USA	The Rocky Mountain Region of Colorado WWTP	Tertiary Effluent			330	
	Las Vegas, NV	USA	-	Tertiary Effluent			970	
	Florida	USA	-	Tertiary Effluent			1200	
Wilson et al. (2013)[231]	Lake Havasu City, Arizona	USA	North Regional WWTP	Final Effluent			2159	
Yang et al. (2004)[232]	Fort Collins, Colorado	USA	Drake WRF	Secondary Effluent			180	
	Fort Collins, Colorado	USA	Drake WRF	Secondary Effluent			130	
Yang et al. (2005)[50]	Fort Collins, Colorado	USA	Drake WRF	Secondary Effluent			390	
Yang et al. (2011)[233]	Gwinnett County, GA	USA	The F. Wayne Hill Water Resources Center	Tertiary Effluent	35	140	80	

	Gwinnett County, GA	USA	The F. Wayne Hill Water Resources Center	Tertiary Effluent	35	150	80	
				Median of Mean Effluent Concentrations			**385**	
				90th Percentile of Mean Effluent Concentrations			**1418**	

Table A2. Estimated environmental concentrations (EECs), predicted no-effect concentrations (PNECs), hazard quotient (HQ), and dilution factor required (DFreq) to meet each hazard quotient for ciprofloxacin, erythromycin, and sulfamethoxazole. PNEC data for resistance selection is obtained by Bengtsson-Palme and Larsson (2016)[234] and Kraupner et al. (2018)[235]. a) HQ and DF_{req} are calculated using EECs equal to the median of reported MECs. b) HQ and DF_{req} are calculated using EECs equal to the 90th percentile reported MECs.

A)

Antibiotic	**N**[1]	**EEC (ng/L)**	**PNEC (ng/L)**	**HQ**	**DF_{req}**
Ciprofloxacin	314[32]	193.2[32]	100[235]	1.932	19.320
Erythromycin	195[33]	107[33]	1000[234]	0.107	1.070
Sulfamethoxazole	49	385	16000[234]	0.024	0.241

B)

Antibiotic	**N**[1]	**EEC (ng/L)**	**PNEC (ng/L)**	**HQ**	**DF_{req}**
Ciprofloxacin	314[32]	8745[32]	100[235]	87.45	874.500
Erythromycin	195[33]	331.5[33]	1000[66]	0.332	3.315
Sulfamethoxazole	49	1418	16000[66]	0.089	0.886

[1] These numbers correspond to the number of data points used to calculate each EEC.

Table A3. The number of the sites that exceeded the safety threshold for ciprofloxacin under monthly streamflow and mean annual flow (MAF); color-coded based on the percentage of the sites that exceeded ARB threshold located on specific Strahler stream order to the total number of WWTPs located on the same stream order.

SSO	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	MAF	# WWTPs
1	2695	2285	1615	2019	2490	2827	3284	3550	3429	3148	2650	2686	2757	4149
2	987	796	595	714	902	1041	1319	1472	1393	1264	1073	981	1007	2274
3	558	431	326	379	462	569	743	864	821	745	608	542	528	2036
4	240	195	148	144	186	233	313	367	355	341	273	241	206	1758
5	105	86	63	66	73	83	111	138	141	132	119	105	80	1279
6	43	38	30	30	28	33	40	52	49	55	48	49	34	912
7	18	14	11	9	10	8	12	16	15	16	15	17	10	468
8	11	9	6	6	6	6	6	6	7	9	8	9	7	248
9	0	0	0	0	0	0	0	0	0	0	0	0	0	121
Total	4657	3854	2794	3367	4157	4800	5828	6465	6210	5710	4794	4630	4629	13245

RANKINGS	**0%-20%**	**20%-40%**	**40%-60%**	**60%-80%**	**80%-100%**

Table A4. The number of the sites exceeded the ABR safety threshold for ciprofloxacin and erythromycin under mean annual streamflow where EEC is modeled as 90th percentile of MECs and median of MECs; color-coded based on the percentage of the sites exceeding ABR threshold located on specific Strahler stream order to the total number of WWTPs located on the same stream order.

	Ciprofloxacin		**Erythromycin**		
SSO	**EEC=90th PC of MECs**	**EEC=Median of MECs**	**EEC=90th PC of MECs**	**EEC=Median of MECs**	**# WWTPs**
1	4137	2757	1035	117	4149
2	2237	1007	289	25	2274
3	1893	528	123	8	2036
4	1332	206	42	4	1758
5	698	80	24	0	1279
6	328	34	13	0	912
7	141	10	7	0	468
8	33	7	6	0	248
9	3	0	0	0	121
Total	10802	4629	1539	154	13245

RANKINGS	**0%-20%**	**20%-40%**	**40%-60%**	**60%-80%**	**80%-100%**

CHAPTER 4: GEOSPATIAL DISTRIBUTION OF WATERSHED VULNERABILITY TO ANTIBIOTIC RESISTANCE IN STREAMS ACROSS THE U.S.

* This chapter is in preparation for submission to *Science of the Total Environment*

4.1 ABSTRACT

The spread of antibiotic resistance threatens human and ecological health. This study incorporates antibiotic point and nonpoint pollution in a geographic information system (GIS) framework to identify the geospatial distribution of watershed vulnerability to antibiotic resistance (AR) occurrence among 4-digit hydrologic unit (HUC-4) watersheds across the U.S. We developed a multimetric index incorporating multiple point and nonpoint sources of antibiotics such as wastewater treatment plants, hospitals, antibiotic prescriptions, manure, and animal feeding operations, which is paired with the hydrologic condition and climate change indicators for each watershed. Our results indicate that watersheds in the Western U.S. are highly vulnerable to antibiotic resistance occurrence, especially within the Upper Mississippi (07) and Great Lakes (04) hydrologic regions. Model predictions suggest less vulnerability to AR occurrence in the Southern and Western regions. In addition, antibiotic nonpoint pollution was found to be the predominant factor impacting the antibiotic resistance vulnerability index among the majority of examined states. These findings provide a scientific foundation for enhancing watershed management in the midwestern U.S., as well as a framework for identifying watersheds at higher vulnerability to AR occurrence and prioritizing the watershed management strategies within identified watersheds across the U.S.

4.2 INTRODUCTION

Antibiotics and antibiotic resistance (AR) have been profiled as contaminants of emerging concern (CECs) due to the potential adverse impacts on ecological and human health[1, 2]. Antibiotic presence in the aquatic environment might further complicate the development and dissemination of AR due to selective pressure on bacteria imposed by antibiotic concentrations[3]. Many agencies warned about the rising risks of AR on human health and economics[4, 5]. Centers for Disease Control and Prevention reported over 2.8 resistance-related infections annually in the U.S., with a death rate of more than 35,000 per year and an economic burden of over $4.6 billion per year to treat infections[6]. In addition, World Health Organization (WHO) has classified antimicrobial resistance (AMR) as the top 10 threats to global public health[5]. The presence of AR in the environment can pose a risk to the biodiversity of the natural microbial community, leading to disruption in ecological function[7]. Despite the significant impacts on human and ecological health, antibiotics and AR are not commonly regulated or/and monitored and lacks effective combat and control strategies mainly due to incomplete knowledge of the occurrence and spread of antibiotics and AR in various environmental matrices[8].

Higher levels of antibiotics and AR have been detected in environments with higher anthropogenic activities, such as agricultural/urban activities, than in nature-dominated environments[9, 10]. Antibiotic and AR concentrations significantly increased near point sources (i.e., wastewater treatment plants [WWTP], hospitals, pharmaceutical production facilities) and nonpoint sources (i.e., agricultural farms and animal feeding operation [AFOs])[11-18]. Municipal effluent is one of the key sources of antibiotics and AR in the aquatic environment, especially due to the incomplete removal of antibiotics and

AR in WWTPs[19]. Antibiotics are considerably used in the production of livestock to cure infections and promote growth [20], leading to manure and wastewater contamination with antibiotics and AR[21]. The elevated level of antibiotics and AR have been detected in surface water and groundwater near pig and poultry farms[22]. Since the application of manure as an agricultural fertilizer represents a common practice, antibiotic and AR residues can travel from soil to groundwater as well as surface water by agricultural runoff[23-25]. Extremely high levels of antibiotics have been reported in the effluent of pharmaceutical manufacturers, up to a level of mg/L [18, 26]. Consequently, the identification of antibiotic pollution sources plays a vital role in establishing control and mitigation strategies.

Hydrologic characteristics and spatiotemporal variations in hydrologic regimes may impact dilution or enrichment of antibiotics and AR in aquatic settings. [27-30]. Bigger-sized streams are generally less sensitive to seasonal flow fluctuations[31,] and their higher instream flow rates result in greater dilution capacity and thus lower antibiotic and AR contamination[27]. In addition to hydrologic conditions, climate change has considerable impacts on the development and spread of AR[32]. Increases in ambient temperature have increased horizontal gene transfer, the main mechanism in the dissemination of AR[33] and bacterial growth rate [34]. For example, MacFadden et al (2018) noted positive associations between the increasing local temperature (average minimum temperature) on AR across the U.S.[35]. In addition, flooding or intense precipitation caused by climate change intensifies agricultural runoff and therefore increases the spread of antibiotics and AR from animal farms and manure treated lands[36]. Furthermore, drought events or/and a climate-driven decrease in instream flow increase the contribution of point sources of antibiotics and AR such as WWTP effluent[36]. Therefore, the impacts of hydrologic characteristics, seasonality, and projected climate change on antibiotic and AR occurrences are important factors in identifying impacted watersheds by antibiotic resistance presence.

Most field studies on antibiotic resistance have been performed by sample collection from certain regions during a specific time[1]. However, sampling efforts are limited to the number of analytes and might under or overestimate antibiotic and AR quantities due to spatiotemporal variations and seasonality in hydrologic characteristics and anthropogenic input[37, 38]. In addition, understanding of AR occurrence lacks a holistic approach to evaluate impacts of antibiotic point and nonpoint pollution, hydrologic conditions, and climate change on the watershed scale (state or national). Multimetric indices (MMIs) have been successfully used to assess watershed health and ecological conditions in Europe and the U.S. by considering a combination of metrics representing multiple anthropogenic disturbances, water quality, biological and hydrologic attributes[39-44]. For example, U.S. Environmental Protection Agency (USEPA) develops a watershed health multimetric index at both regional and state levels as a part of the healthy watersheds program to systematically compare a comprehensive set of ecological conditions across watersheds[45]. This study incorporates antibiotic point and nonpoint pollution alongside hydrologic and climate change indicators in a geographic information system (GIS) framework to rank watersheds from most to least vulnerable to AR occurrences at HUC-4 watersheds across the U.S. using EPA-developed assessment methodology.[45-47]

4.3 MATERIALS AND METHODS

4.3.1 CANDIDATE METRICS AND INDEX OVERVIEW

Watershed vulnerability index to AR occurrence was developed for 209 hydrologic subregions (4-digit HUC or HUC-4) to identify watersheds that are most impacted by anthropogenic inputs containing antibiotics across the U.S. The approach derived from the U.S. EPA's Healthy Watersheds Integrated Assessment[45-47]; and was altered to consist of seven metrics within three index categories investigating various aspects of anthropogenic stressors, hydrologic conditions, and climate change (as shown in Table 1). Collectively, the proposed metrics are expected to provide an estimate for potential AR impacts and distinguish least- from most-impacted watersheds.

Antibiotic point and nonpoint pollution were characterized by the following metric for each HUC-4 watershed across the U.S.; aggregated wastewater effluent flow normalized by watershed area as WWTP, aggregated hospital effluent flow normalized by watershed area as hospital, a total number of antibiotic prescriptions normalized by watershed area as use pattern, a total amount of antibiotic used in food-producing animals (cattle, swine, and chicken) normalized by watershed area as animal-use and aggregated manure treated areas normalized by watershed area. To characterize watershed hydrologic conditions, dam storage ratio was used indicating the potential for altered streamflow due to upstream impoundments. To investigate the impacts of climate-driven changes on AR dissemination, the projected minimum temperature was used as a climate change metric.

4.3.2 DATA COLLECTION

The framework for the model is ArcGIS 10.8.1. HUC-4 watershed data was extracted from USGS Watershed Boundary Dataset Data Model (V2.3)[48]. Additionally, the U.S. boundary was obtained from the 2018 Census Bureau's MAF/TIGER geographic database[49]. Locations and present design flow of 13,245 WWTPs across the US were obtained from the 2012 Watersheds Needs Survey (CWNS) developed by US EPA[50]. Location details and the number of beds used were extracted from Homeland Infrastructure Foundation-Level data (HIFLD)[51]. Dasymetric population for the US was obtained from EPA EnviroAtlas[52], where the 2010 population has been relocated from census block to 30-meter pixel considering slope and landcover. Antibiotic prescriptions rate per 1,000 persons per state for 2018 was retrieved from the Outpatient Antibiotic Stewardship annual report[53]. Manure treated areas and a total number of cattle, swine, and chicken per HUC-6 watershed were extracted from the 2017 census of agriculture reported by the U.S. Department of Agriculture and aggregated to HUC-4 boundary[54]. Species-specific annual antibiotic totals used in major food-producing animals for 2018 were provided by Food and Drug Administration (FDA) [55]. Dam locations and impounded water volumes were provided by the 2018 National Inventory of Dams (NID)[56]. Estimated mean annual predevelopment streamflow was obtained for each HUC-4 watershed outlet from NHDPlus V2[57]. Change between future (2050-2074) and historical (1950-2005) annual minimum temperature for U.S. HUC-4 watersheds was extracted from USGS National Climate Change Viewer (NCCV), where the minimum temperature projection is derived from 30 Coupled Model Intercomparison Project 5

(CMIP5) climate models downscaled to an 800-m grid for high Representative Concentration Pathway (i.e., RCP 8.50 emissions scenario[58].

4.3.3 DATA PROCESSING AND CALCULATION OF METRIC VALUES

Hospital effluent flow was calculated for 7095 hospitals across the U.S. based on water usage [59, 60]. Hospital water usage was estimated by multiplying the number of beds for a given hospital by the typical water usage values per bed (315 gal/bed/day)[61]. The hospital effluent flow was then calculated to be equal to 80% of total water use[59, 60]. Then, hospital effluent flow was aggregated for HUC-4 watersheds by spatially joining each HUC4-watershed to hospitals and applying a merge rule of sum for effluent flow. In the same approach, WWTP effluent flow was aggregated for HUC-4 watersheds.

Population residing within HUC-4 watersheds was summarized by aggregating the dasymetric data to the watershed boundary using the Zonal Statistics tool. Antibiotic prescriptions rate per 1,000 persons were converted into a raster dataset. The zonal operation was performed to calculate the prescription rate per watershed equal to the mean of raster values. Then, the prescription rate at the watershed level was multiplied by population to yield total antibiotic prescriptions per watershed. *Appendix*, Figure A5 indicates the total antibiotic prescription per HUC-4 watershed across the U.S.

Metrics indicating antibiotic nonpoint pollution were estimated for each HUC-4 watershed. Manure treated areas and a total number of cattle, swine, and chicken per HUC-6 were aggregated by the 4-digit watershed boundary to summarize these values by HUC-4[54]. The amount of antibiotics used per animal per year for a given species was estimated as the total amount of antibiotics used in a specific species [55] divided by the total number of that species in the U.S.[54] The amount of antibiotics used per individual animal in 2018 for cattle, swine, and chicken is 61.68, 41.26, and 0.71 g/ animal, respectively (*Appendix*, Table A5). These quantities were multiplied by the total number of a specific species per HUC-4 watershed to quantify the total antibiotic used in major food-producing animals per watershed. *Appendix*, Figure A6 indicates total antibiotic use by animals per HUC-4 watershed across the U.S. Lastly, metric values of antibiotic pollution were normalized by dividing the metric value for a given watershed by the corresponding watershed area to obtain the density of metrics and to avoid overestimation due to higher areas of watersheds.

The dam storage ratio of each HUC-4 watershed was quantified as the volume of water impounded by dams in the watershed divided by annual flow volume at the watershed outlet. HUC-4 watershed outlet (pourpoints) was set to the stream within the watershed with the highest mean annual flow and was verified visually to ensure it pours to the downstream watershed defined by NHDPlus V2[57]. The total volume of dams per watershed was quantified by aggregation of dam volume to watershed boundary via the Spatial Join Tool.

Table 1. Index categories and metrics to assess watershed vulnerability to AR occurrence.

<table>
<tr><th>Sub-index Name</th><th>Sub-index Category</th><th>Metric</th></tr>
<tr><td rowspan="5">Antibiotic point and nonpoint pollution</td><td rowspan="2">Nonpoint pollution</td><td>Manure treated areas[1]</td></tr>
<tr><td>Estimated antibiotic use in cattle, swine, and chicken[1]</td></tr>
<tr><td rowspan="3">Point pollution</td><td>WWTP effluent flow[1]</td></tr>
<tr><td>Hospital effluent flow[1]</td></tr>
<tr><td>Antibiotic prescitiption[1]</td></tr>
<tr><td>Watershed hydrologic condition</td><td>Anthropogenic watershed condition sub-index</td><td>Dam storage ratio</td></tr>
<tr><td>Projected climate change</td><td>Climate change sub-index</td><td>Projected minimum temperature change</td></tr>
</table>

[1] Normalized by area of HUC-4 watersheds.

4.3.3 MULTIMETRIC INDEX (MMI) CALCULATION INDICATING U.S. WATERSHED VULNERABILITY TO AR OCCURRENCE

The continuous metric scoring method suggested by EPA[45-47] was used to calculate resistance vulnerability MMI due to higher consistency and better responsiveness, and less subjectivity in comparison to a discrete scoring system [44, 47, 62]. All selected metrics have similar directionality in regard to their impacts on watershed vulnerability to AR occurrence. Thus, the metric scores were calculated according to Equation (4). The equal importance of metrics and sub-indices was assumed (i.e., weighting was not applied) due to the complexity of the system and the lack of studies investigating the impacts of each metric. Upon completion of metric scores, sub-index scores for the attribute with more than one indicator (i.e., sub-index of antibiotic point and nonpoint pollution) were calculated based on Equation (5), where the number of metrics within a given sub-index is defined as n. Finally, sub-indices were averaged to calculate the index of watershed vulnerability to AR occurrence according to Equation (6), where m is equal to 3, the number of sub-indices. The final scores of the index and sub-indices of watershed vulnerability to AR occurrence were normalized to range between 0 to 100 for better communication purposes. Low scores are associated with high vulnerability to AR occurrence.

$$Metric\ score = \left(1 - \frac{Observed\ metric\ for\ given\ watershed}{Maximum\ metric\ value\ for\ all\ watershed\ in\ the\ US}\right) * 100 \qquad (4)$$

$$Sub - index\ score = \frac{\sum_{i=1}^{n} MetricScore_i}{n} \qquad (5)$$

$$Final\ index\ score = \frac{\sum_{j=1}^{m} Sub\_indexScore_j}{m} \qquad (6)$$

4.4 RESULTS AND DISCUSSIONS

4.4.1 GEOSPATIAL VARIATIONS IN POINT AND NONPOINT ANTIBIOTIC POLLUTION

Antibiotic pollution estimates to watersheds across the U.S. were grouped by antibiotic loading (nonpoint vs. point) and aggregated at the HUC-4 scale to evaluate variations in regional contamination. Estimates for nonpoint antibiotic contributions included manure application and livestock antibiotic use. Approximately 17900 tons of antibiotics were sold and distributed in 2011-2012 across the U.S., which over 80% were consumed by food-producing animals (14600 tons)[63, 64]. According to the results, the highest amount of antibiotics in animals have been used in watersheds within the Midwestern and Southeastern U.S. due to the largest livestock industry, highlighting the potential for watershed vulnerability due to antibiotic loadings from animal feeding operations. Furthermore, the highest manure application has been observed in watersheds across the Midwestern U.S. due to intense crop farms in the region. The spatial distribution of individual scores of manure treated areas and antibiotic use in animals is shown in *Appendix*, Figure A7.

Figure 5a indicates that watersheds located in the Midwestern U.S. are dominantly at higher risk of antibiotic nonpoint pollution loading into surface water. The most widely used antibiotic classes in animals are tetracyclines, penicillins, and macrolides from medically important antibiotics alongside ionophores from non-medically important antibiotics[55]. Therefore, these antibiotic classes along with the potential for antibiotic microbial resistance are expected to be heightened in hydrologic subregions of the Upper Mississippi, Great Lakes, Mid-Atlantic, South Atlantic, North

Ohio, Lower Missouri, Arkansas-Red-White, and Central California. In response, regional watershed management strategies should prioritize mitigating risks from antibiotic nonpoint pollution.

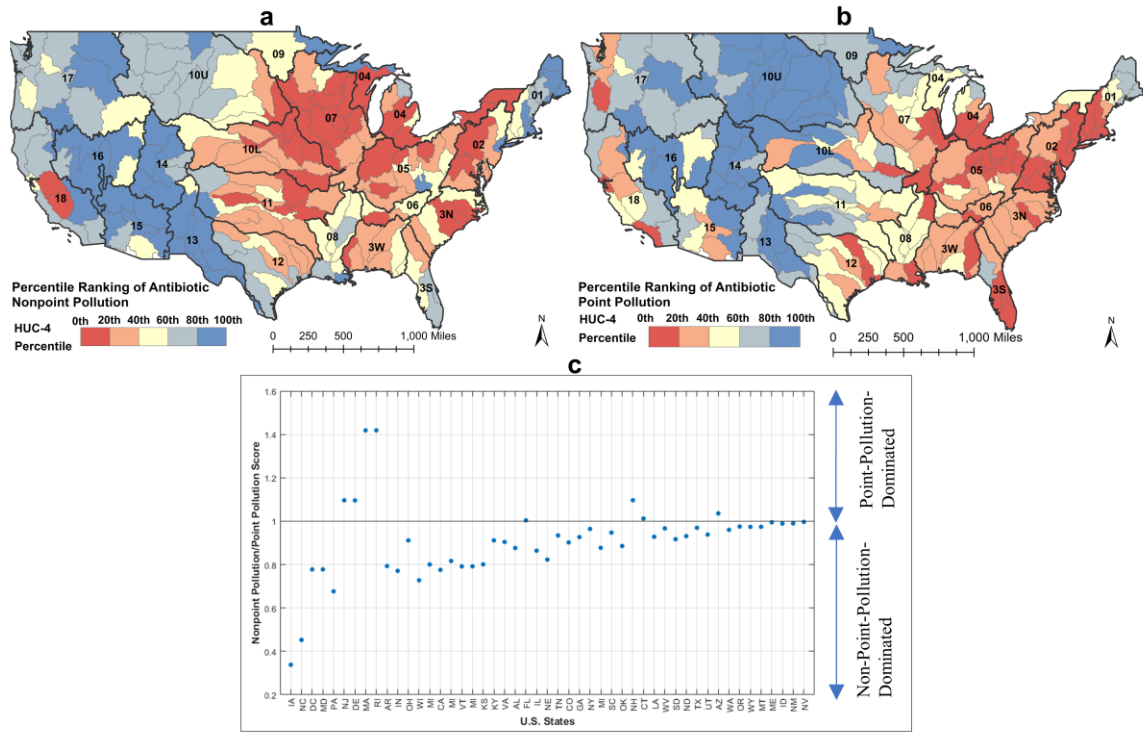

Figure 5. Spatial distribution of watersheds dominated by point and nonpoint pollution. a) percentile rank of nonpoint pollution (i.e., use in animal feeding operation and manure application), b) percentile rank of point pollution (i.e., municipal WWTP, hospital and oral prescription; and c) antibiotic-impacted watersheds, classified by states, in the order from high to low antibiotic pollution loading, and relative contribution of point vs nonpoint pollution, compared to other states.

Scores of antibiotic point pollution were calculated based on aggregated values for municipal and hospital wastewater, and total antibiotic prescriptions. Urban watersheds with high population density are most impacted by antibiotics discharged from treated effluent of WWTPs and hospitals into surface water. These watersheds are located across the Northeast, Southeast, and Midwest in the hydrologic subregions of the Mid-Atlantic, Ohio, and Northern South Atlantic. In addition, the highest antibiotics have been prescribed in the Southeastern U.S., with penicillins and macrolides as the most commonly prescribed antibiotic classes. The spatial distribution of individual scores of aggregated municipal wastewater flow, aggregated hospital wastewater flow, and total antibiotic prescriptions are shown in *Appendix*, Figure A8. Once compounded, Figure 5b indicates that watersheds within the eastern U.S have a relatively higher score of antibiotic point pollution. Especially, within urban watersheds in the vicinity of Chicago, Detroit, Cleveland, Boston, New York City, Los Angeles, and San Diego. Therefore, advanced treatment technologies and effluent monitoring programs should be prioritized in these watersheds.

Scores for antibiotic point and nonpoint pollution were then averaged to calculate the sub-index score for antibiotic pollution. Figure 6a shows that the majority of watersheds ranked below the 40^{th} percentile is located in Midwestern and Northeastern U.S. within hydrologic subregions of the Upper Mississippi, Great Lakes, Mid-Atlantic, and Ohio. Watersheds with lower percentile rank are at a higher risk of antibiotic point and or nonpoint pollution. In spite of not directly estimating index scores for Lake Erie, Lake Huron, and Lake Michigan, we expect near-shore impacts from upstream antibiotic pollution.

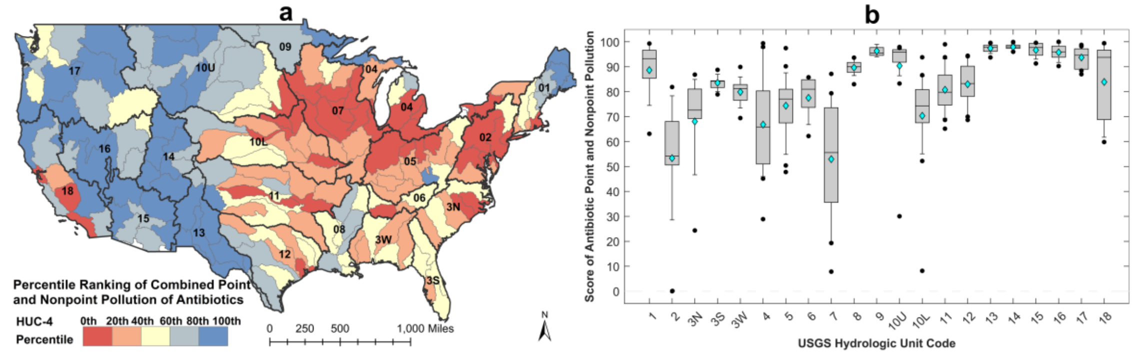

Figure 6. Sub-index of antibiotic pollution, a) Spatial distribution of percentile rank of combined point and nonpoint pollution of antibiotic, b) box and whisker plot of sub-index scores of point and nonpoint pollution has shown for the associated 2-digit hydrologic unit. Top and bottom of the box: 75th and 25th percentiles, respectively; top and bottom of whisker:90th and 10th percentiles, respectively; line inside the box:50th percentile (median); diamond (♦): average.

Identifying antibiotic point and nonpoint pollution and their relative contribution is a vital part of steps toward understanding and mitigating occurrence. Figure 5c indicates the most to the least impacted watersheds by antibiotic pollution, classified by states, alongside the relative contribution of point vs nonpoint pollution on the sub-index of antibiotic pollution. Nonpoint pollution is modeled to be significantly impactful on the sub-index score and thus on potential AR occurrence among the majority of states. For example, antibiotic use in animal feeding operations and manure application on agricultural land significantly contribute to Iowa and North Carolina being modeled as the first and second impacted by antibiotic pollution across the U.S. Indeed, Iowa and North Carolina have the greatest number of swine CAFOs, which account for 44% of total swine production in the U.S. [54]. Figure 7 indicates the geospatial distribution of point and nonpoint antibiotic pollution within six highly impacted states, including Iowa, Wisconsin, Ohio, Indiana, North Carolina, Pennsylvania, and New Jersey. Both point and nonpoint pollution attribute significantly to antibiotic loading to surface water in Iowa, Wisconsin, Ohio, Indiana, North Carolina, Pennsylvania, whereas point pollution is the main pathway for antibiotics within New Jersey waterways.

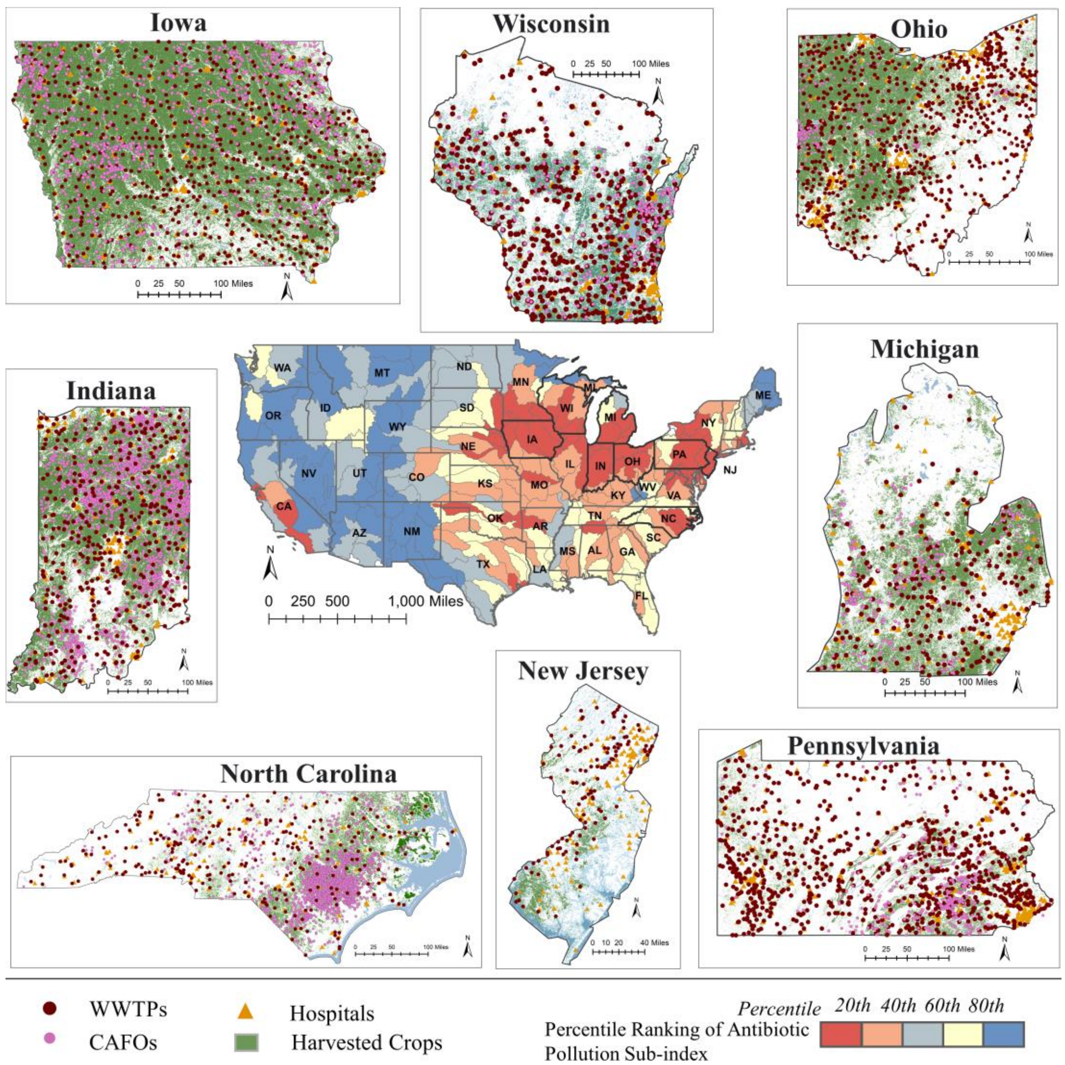

Figure 7. Geospatial distribution of antibiotic point and nonpoint pollution in highly impacted states by antibiotic pollution.

4.4.2 INFLUENCE OF HYDROLOGIC CONDITION AND CLIMATE CHANGE ACROSS THE U.S.

Dams are considered as one of the significant stressors on streams, altering the natural flow, stream temperature, and downstream water quality[65, 66]. Dam storage ratio has been used to incorporate the impact of the anthropogenic hydrologic conditions on watershed vulnerability to AR occurrence. Figure 8a indicates that watersheds located in the western U.S. are generally impacted by dams due to a higher dam storage ratio, highlighting higher natural flow alteration within western watersheds including Great Basin, California; Upper and Lower Missouri; Upper and Lower Colorado; and South Atlantic.

Previous studies have shown that antibiotic resistance prevalence has more rapidly risen in U.S. regions and European countries with warmer minimum temperature[35, 67]. The projected minimum temperature change will adversely impact antibiotic resistance in watersheds in the northern United States. Upper Mississippi, Great Lakes, Northeast, Souris-Red-Rainy, Upper Colorado, and Great Bain will endure the highest projection minimum temperature change, potentially adding to the vulnerability of these watersheds to antibiotic resistance occurrence due to climate-driven temperature changes (as shown in Figure 8b).

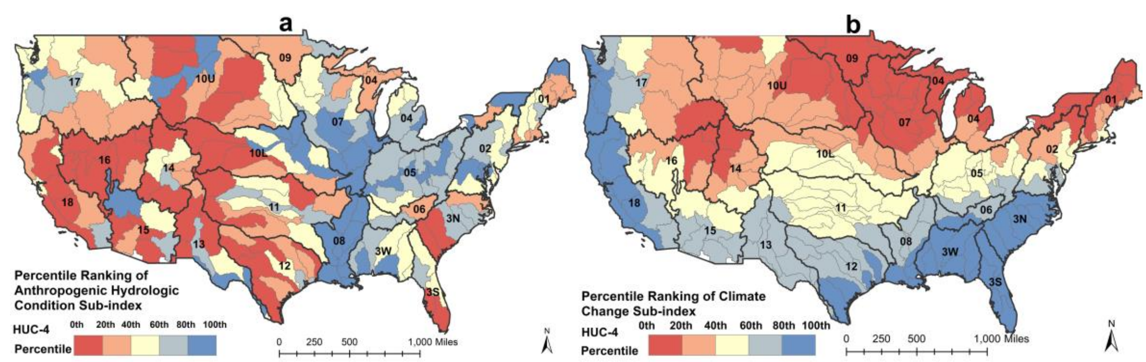

Figure 8. Trends in the geospatial distribution of a) hydrologic conditions based on metric of dam storage ratio, b) climate change based on changes in mean annual minimum temperature between future (2050-2074) and historical (1950-2005), color-coded using quantile classification method.

4.4.3 WATERSHED VULNERABILITY TO AR OCCURRENCE

Watershed vulnerability to AR occurrence is shown in Figure 9, as the aggregation of the antibiotic point and nonpoint pollution, hydrologic condition, and climate change subindices. Watersheds located in Midwest are dominantly at higher risk of AR occurrence. Figure 9b indicates that hydrologic subregions of Upper Mississippi (07), Great Lakes (04), Souris-Red-Rainy (09), and Mid-Atlantic (02) have the lowest mean index score, highlighting a higher potential presence of antibiotic and AR mainly due to the significant point and nonpoint pollution coupled with higher projected minimum temperature change. The high density of animal feeding operations and crops production plays an important role in the dissemination of antibiotics and AR in these watersheds. In addition to agricultural pollution, urban watersheds in the Midwest such as Chicago metropolitan, Detroit metropolitan, and Twin cities significantly contribute to antibiotic loadings in surface water. Consequently, Lake Michigan, Lake Erie, and Lake Huron are expected to be vulnerable to AR occurrence from upstream anthropogenic activity. Furthermore, urban watersheds with a high population such as Greater Los Angles and New York Metropolitan Area are vulnerable to AR occurrence from antibiotic point pollution. Our vulnerability index shows lower vulnerability AR occurrence in the western and southern U.S. as the majority of the watersheds ranked below the 60th percentile.

Our results are consistent with the results from EPA’s index of watershed integrity (IWI) and monitoring results of antimicrobial resistance genes from EPA’s National Rivers and Streams Assessment (NRSA)[68-70]. IWI incorporates 6 watershed components into watershed stressors to indicate the capacity of watersheds to support ecological processes[68, 69]. Thus, low integrity means that watersheds in incapable of maintaining ecological processes required for biodiversity[68, 69]. IWI has reported low integrity in the Midwestern U.S., high integrity in the Western U.S., and intermediate integrity in Southern and Eastern U.S.[68, 69] Correlations have been observed between results of IWI and EPA's antimicrobial resistance genes monitoring across the U.S., in which high concentrations of *intl1*, *sul1*, *tetw,* and *blaTEM* have been observed in the Midwestern U.S.[70]

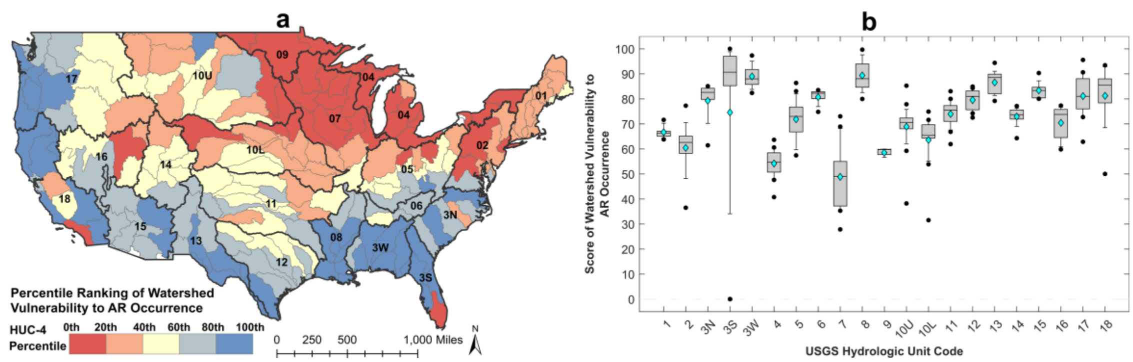

Figure 9. Watershed vulnerability to AR occurrence. a) Spatial distribution of watershed vulnerability to AR occurrence, color-coded using quantile classification method. b) Distribution of HUC-4 index scores of watershed vulnerability index to AR occurrence shown for the associated 2-digit hydrologic unit. Top and bottom of the box: 75th and 25th percentiles, respectively; top and bottom of whisker:90th and 10th percentiles, respectively; line inside the box:50th percentile (median); diamond (♦): average.

4.4.4 LIMITATIONS

Due to the scale of the analysis, generalizations were required, and data was limited to datasets available at the national scale. Dam storage ratio which was used as a hydrologic condition indicator is a coarse estimate that does not represent instream flow conditions, seasonal fluctuations in streamflow, and other significant hydrologic processes that impact antibiotic concentrations and AR development and spread through the aquatic environment. Although more sophisticated analysis investigating hydrologic conditions at the national level is a challenging task, future work may entail indicators of receiving streamflow and seasonality when feasible. However, the dam storage ratio was assumed to be sufficient for the national-wide investigation of watershed vulnerability to antibiotic and AR exposure. Antibiotic and ARG emissions from point and nonpoint sources into surface water were not quantified in this study. This is in part due to the absence of national datasets detailing sources and their related attributes such as geocoded CAFO database with information regarding animal types, and allowable counts; total antibiotic use by a human per antibiotic classes in the U.S.; and antibiotic consumption rates in hospitals.

4.5 CONCLUSIONS

In this study, we assessed watershed vulnerability to AR occurrence across the U.S. through the development of a multimetric national index considering the cumulative impact of point and nonpoint pollution (i.e., WWTPs, hospitals, antibiotic prescription, manure application, and animal feeding operations) as well as hydrologic conditions and projected climate change. In this study, we did not quantify emissions from sources, but rather we identified watershed vulnerability based on the assumption that higher potential sources will lead to elevated antibiotic and AR concentrations in the aquatic environment. Our results indicate that watersheds in the western U.S. are at the highest risk of exposure to antibiotics and AR due to the densified urban and agricultural activities. Our approach can be applied to local and national hydrologic scales to examine the relative differences of potential antibiotic and AR occurrences among watersheds. This approach can be used to inform decision-makers and to design surveillance and mitigation program at multiple scales, especially when coupled with site-level information regarding antibiotic and AR occurrences. In addition, the vulnerability index can be readily applied and improved as advanced data become accessible.

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4.7 APPENDICES

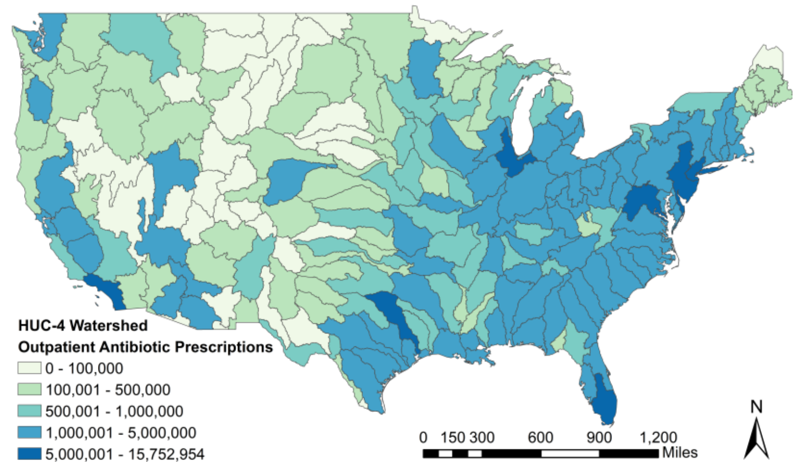

Figure A5. Spatial distribution of total antibiotic prescriptions per HUC-4 watersheds across the U.S.

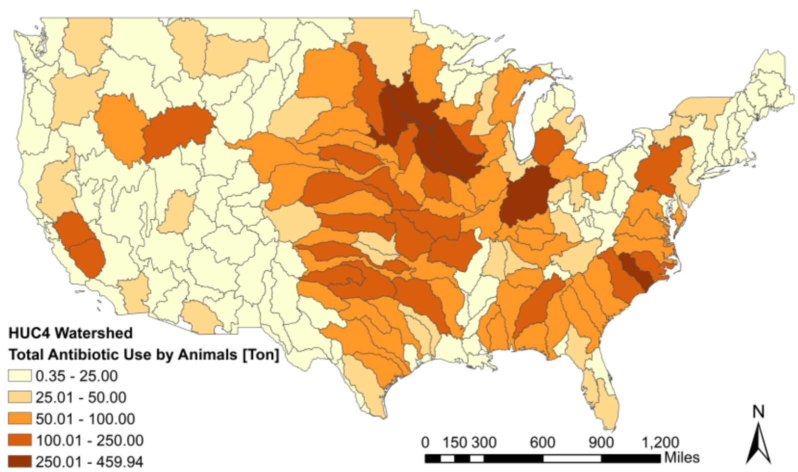

Figure A6. Spatial distribution of total antibiotic use by animals (swine, chicken, cattle) per HUC-4 watersheds across the U.S.

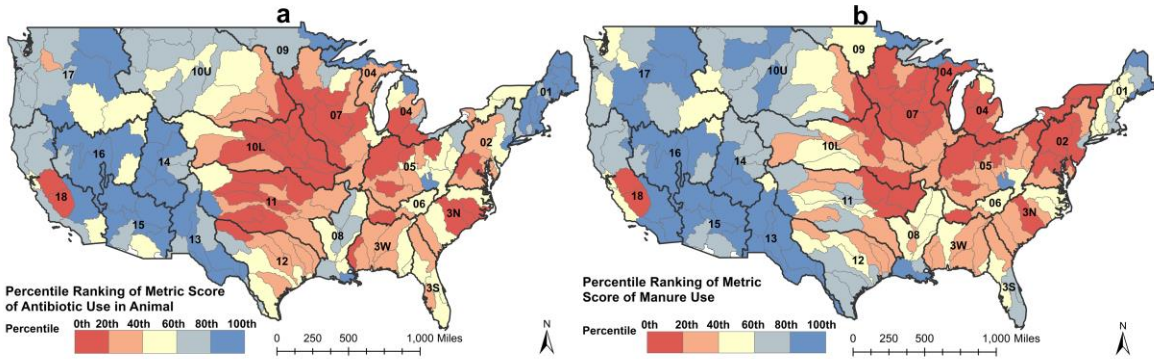

Figure A7. Percentile ranking of metrics indicating nonpoint pollution per HUC-4. a) antibiotic use in animals, b) manure treated area.

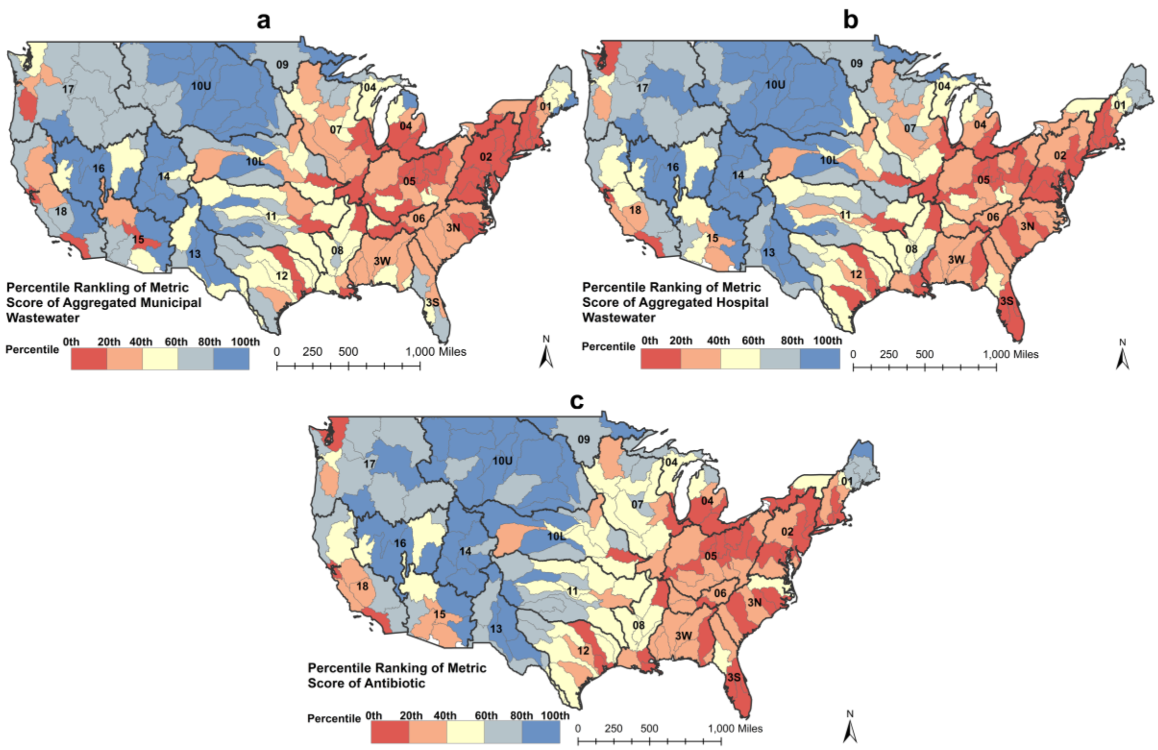

Figure A8. Percentile ranking of metrics indicating point pollution per HUC-4, a) aggregated municipal wastewater flow, b) aggregated hospital wastewater flow, and c) total antibiotic prescriptions.

Table A5. Values used to estimate antibiotic use per animal per species in the U.S.

NOT MEDICALLY IMPORTANT[14]		**MEDICALLY IMPORTANT**[14]		**GRAND TOTAL**[14]			
Species	**Estimated Annual Totals (kg)**	**Species**	**Estimated Annual Totals (kg)**	**Species**	**Estimated Annual Totals (kg)**	**Total Number in the U.S.**[236]	**Antibiotic use per Animal (g)**
Cattle	3,246,667	Cattle	2,529,281	Cattle	5,775,948	93,305,560	61.90
Swine	404,343	Swine	2,582,399	Swine	2,986,742	72,273,569	41.33
Chicken	1,315,354	Chicken	192,964	Chicken	1,508,318	2,118,283,043	0. 71
Turkey	310,426	Turkey	644,921	Turkey	955,347	-	-
Other	2,308	Other	239,694	Other	242,002	-	-
Total	5,279,098	Total	6,189,260	Total	11,468,358	-	-

CHAPTER 5: AN INTEGRATIVE ASSESSMENT OF ANTIBIOTIC CONCENTRATIONS IN NORTH CAROLINA WATERSHEDS

* This chapter is in preparation for submission to *Environmental Research*

5.1 ABSTRACT

Improved surveillance of antibiotic resistance throughout the environment is an important aspect of preventing and controlling the threats posed to human and ecological health. Field investigations are often limited by resources and time. Here we present an approach that integrates modeling and field sampling as a step in overcoming these limitations. In doing so, environmental modeling estimates informed a field study that quantified antibiotic concentrations in surface water. A GIS-based model was used to identify the most-impacted watersheds by multiple point and nonpoint pollution of antibiotics to be further investigated by field in-stream sampling. Four commonly detected antibiotics including azithromycin, ciprofloxacin, sulfamethoxazole, and trimethoprim were assessed at modeled impacted sites and respective reference sites. In addition, we used hot spots analysis to determine counties with intense hot spots of antibiotic-impacted watersheds and investigate the racial and socioeconomic status of identified counties. Our modeling results suggest that low-income and less educated communities are more impacted by potential risks associated with antibiotic sources. In addition, antibiotic concentrations were significantly higher at impacted sites, compared to the reference sites ($p < .001$). This study can be employed by researchers, planners, policy, and decision-makers for optimal site selection for the assessment of antibiotic resistance occurrence.

5.2 INTRODUCTION

Anthropogenic activities are the main source of antibiotic and antibiotic resistance (AR) in surface water. Instream antibiotic and AR have been correlated with upstream landscape features such as wastewater treatment plants (WWTPs) and concentrated animal feeding operations (CAFOs)[1, 2]. Elevated levels of antibiotics and AR have been documented in hospital sewage[3]. However, there is a discrepancy with the extent of hospital sewage contributions towards antibiotic and AR loading to WWTPs in the literature[4-6]. In addition to source contributors, instream flow conditions significantly impact the distribution of antibiotics and AR in the surface water[7]. For example, LaPara et al (2015) investigated the impact of multiple WWTP discharges on ARG concentrations in the receiving stream (Upper Mississippi River)[8]. Despite the high quantities of ARGs within wastewater discharges, a negligible impact was found on the ARG level downstream of WWTPs primarily due to the high instream flow of the Upper Mississippi River and the resulting dilution of ARGs within the river, even during the dry season[8]. In addition, temporal fluctuation and seasonality in consumption rate, anthropogenic activities, and precipitation play an important role in environmental antibiotic concentrations and AR prevalence[9-13].

Field studies have been investigated antibiotics and AR occurrences in the urban and rural watersheds across North Carolina[14-17]. Higher antibiotic concentrations and slight increases in ARG abundance were reported downstream of WWTPs in the urban watershed of North Carolina[14]. Gray et al (2019) assessed antibiotic occurrences and the impact of seasonality on antibiotic concentrations in rural surface water and drinking water wells in Piedmont of North Carolina, wherein antibiotics were frequently detected with concentration ranges within up to 1,740 ng/L in surface and groundwater[15]. North Carolina is the second-largest swine producer in the U.S. Therefore, watershed vulnerability to antibiotic and AR exposure from intensive livestock operations is expected[18]. For example, antibiotic-resistant E. coli have been detected in groundwater wells near swine farms in eastern NC[16]. In addition, Livestock-associated *methicillin-resistant Staphylococcus aureus* (LA-MRSA) was observed in employees from CAFOs but not in employees from antibiotic-free animal operations in North Carolina[17].

Environmental modeling is a powerful tool that can be used as a holistic approach to enhance the understanding of potential antibiotic pollution, their prevalence, and relative contributions. For example, Zhang et al (2015) estimated the cumulative antibiotic emission rate for all river basins in China and predicted antibiotic concentrations for 36 compounds in soil, water, and air using the level 3 fugacity model, wherein predicted concentrations were consistent with the measured environmental concentration of antibiotics and resistant bacteria[19]. The results of these modeling efforts can be integrated into field study and watershed management. For example, the Ireland Environmental Protection Agency (EPA) identified the clusters of antibiotic resistance sources and potential dissemination routes in the surface water using a Geographic information system (GIS) model for sampling site selections and informed decision making[20].

This study integrates environmental modeling into a field study to quantify antibiotics in surface water within watersheds that are modeled as the most impacted by anthropogenic inputs containing antibiotics. To address the discrepancy in literature, we examined the influence of hospital sewage on antibiotics content in the aquatic environment by estimating the magnitude of hospital sewage flow to the present design flow of WWTP receiving hospital sewage. Antibiotic occurrences within North Carolina watersheds were investigated by downscaling a previously developed antibiotic pollution index among NC watersheds. Specific sites are selected based on the index for further exploration through a field study. To establish a better understanding of the impact of antibiotic pollution on their occurrences within watersheds, a field study quantifying selected antibiotics was conducted in the top three most-impacted watersheds in NC.

5.3 MATERIAL AND METHODS

5.3.1 QUANTIFICATION OF HOSPITAL CONTRIBUTIONS TO RECEIVING WWTP DISCHARGE

To investigate the role of hospital effluent as a source of antibiotics and AR in the sewage system, the magnitude of hospital discharges into WWTPs was quantified by integrating hospital discharges into a state-level wastewater model of North Carolina using ArcGIS 10.7.1. Hospital location and number of beds were extracted from Homeland Infrastructure Foundation-Level data (HIFLD)[21]. Hospital water usage was estimated by multiplying the number of beds by the typical water usage values per bed (315 gal/bed/day)[22] and hospital effluent flow was calculated to equal 80% of water usage for 147 hospitals across NC [23, 24]. Hospitals were spatially connected to WWTPs by using the closest facility analysis tool within ArcGIS where road networks are used as a proxy for the sewer system[25]. Road network datasets were created using NC detailed streets dataset from ArcGIS StreetMap North America[26].

To confirm hospitals are connected to the correct receiving WWTPs, elevation data was utilized to verify that the appropriate connections have been made. Since the public wastewater collection system is mainly composed of gravity sewer lines[27], a positive elevation difference is expected between hospitals and receiving WWTPs. Therefore, the verification process was done by comparing the elevation of hospitals to the elevation of the modeled receiving WWTPs with the assumption that hospital elevation should be higher than WWTP elevation. This approach is particularly applicable in cases where several WWTPs are located in close proximity to a hospital. Seamless 1/3 arc-second Digital Elevation Model (DEM) layers were provided elevation data for the analysis[28]. Once receiving WWTPs identified, aggregated hospital sewage received by a given WWTP was divided by WWTP design capacity.

5.3.2 INTEGRATED FIELD STUDY DESIGN

The GIS-based model was used to identify the most-impacted watersheds by antibiotic pollution to be further investigated by field in-stream grab sampling centered in NC streams. The selection of sampling sites was based on the antibiotic pollution index. Initially, three metrics were considered to calculate index scores of antibiotic pollutions. These metrics are aggregated WWTP effluent flow per watershed, aggregated hospital sewage flow per watershed, and total antibiotic use in CAFOs per watershed. However, hospital sewage flow was removed based on our results from the investigation of hospital influence on antibiotic load on WWTPs (detailed discussion can be found in Sections 5.3.1). Metrics used in index calculation are shown in Table 2. Index of antibiotic pollution was framed at the 10-digit hydrologic unit code (HUC-10) scale using ArcGIS 10.7.1. Data on CAFO locations and capacities were obtained from the North Carolina Department of Environmental Quality[29]. Antibiotic use per animal in a given species was calculated based on the approach explained in Chapter 4. Total antibiotic use by CAFOs per HUC-10 watershed was quantified equal to the number of allowable animals per species in a given CAFOs multiplied by antibiotic use per one animal for each species. Then, aggregated WWTP effluent flow per watershed and total antibiotic use in CAFOs per watershed were normalized by the area of the respective watershed. Metric scores of point sources (WWTPs) and nonpoint sources (CAFOs) were calculated according to Equation 7. Lastly, two metric scores were averaged to yield in final index scores of antibiotic pollutions for 360 HUC-10 watersheds across the NC.

$$Metric\ score = \left(1 - \frac{Observed\ metric\ for\ given\ watershed}{Maximum\ metric\ value\ for\ all\ watershed\ in\ the\ US}\right) * 100 \tag{7}$$

Table 2. Sub-index categories and metrics to rank most to least impacted watersheds by antibiotic sources.

Sub-index Name	**Sub-Index Category**	**Metric**
Antibiotic pollution	Nonpoint pollution sub-index	WWTP effluent flow normalized by HUC-10 area
	Point pollution sub-index	Estimated antibiotic use in cattle, swine, and chicken normalized by HUC-10 area

Site selection for the field study was determined by the outcome of the multimetric index described above. The top three model estimates for most-impacted watersheds were selected and final sampling sites were identified based on visual inspection and site availability. The three modeled most-impacted HUC-10 watersheds by antibiotic pollution are Six Runs Creek (*HUC 0303000605*), Sugar Creek (*HUC 0305010301*), and Headwaters Northeast Cape Fear River (*HUC 0303000702*). Figure 10 indicates three watersheds that are modeled to be most impacted by antibiotic pollution and respective sampling locations. For each impacted watershed, four sampling sites were selected, which includes three targeted most-impacted sites and one site predicted to experience less impact from upstream sources (total sampling site equal to 12). Details of the exact location of sampling sites can be found in *Appendix*, Table A6. Samples were collected from the twelve sites from a minimum of 0.25 m below the water surface at each site using a water sampler in September 2021. Samples were kept in coolers filled with icepacks while being transferred to the lab and they were stored in the fridge at 4ºC upon arrival.

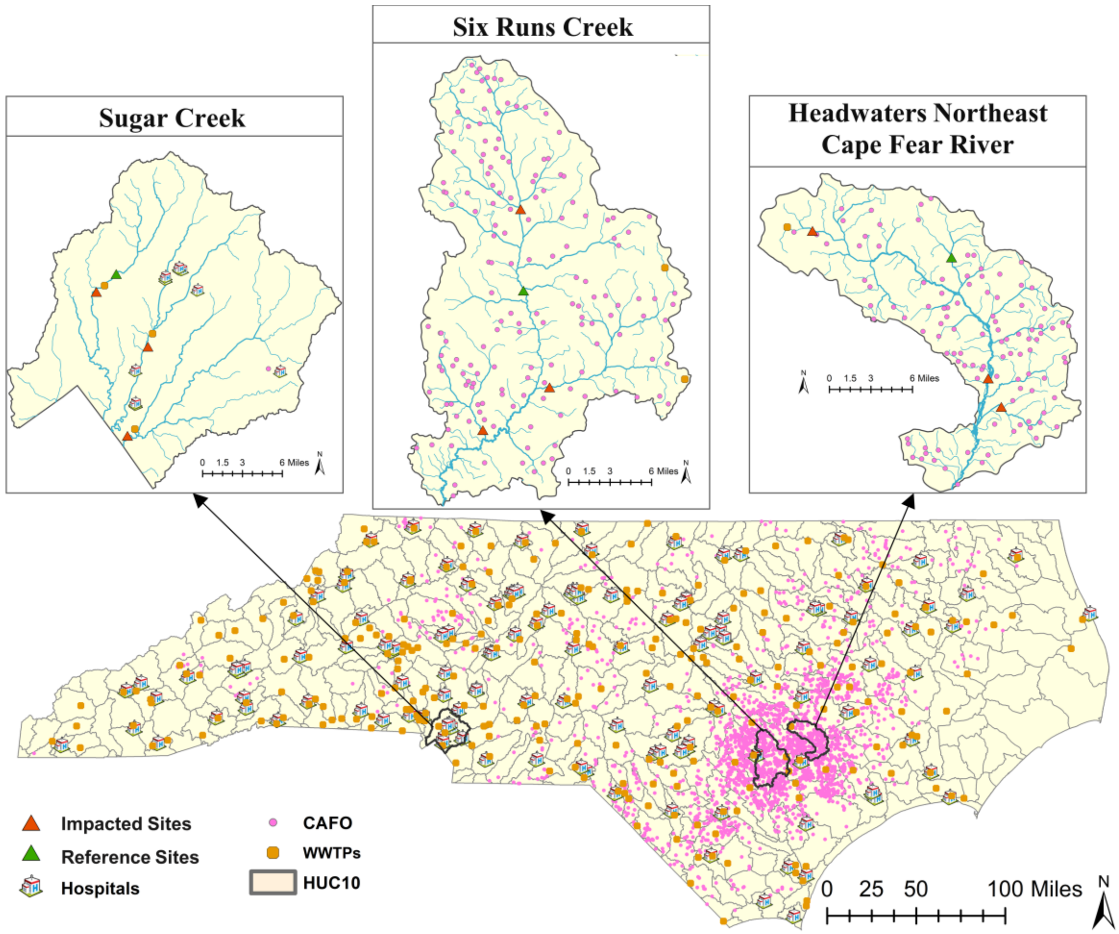

Figure 10. North Carolina HUC-10 watersheds that are modeled to be most impacted by antibiotic and antibiotic resistance sources, and respective selected sampling sites.

5.3.3 SAMPLING AND CHEMICAL WATER QUALITY ANALYSIS

Environmental concentrations for a suite of physical and chemical water quality parameters were analyzed at 12 sites. Samples from selected sites were collected in 1 L acid-washed and baked amber glass bottles. Duplicate samples were collected from each selected site to ensure quality assurance and quality control (QA/QC). Samples were analyzed for four commonly detected antibiotics in surface water, azithromycin, ciprofloxacin trimethoprim, and sulfamethoxazole. In addition to targeted antibiotics, sucralose as a wastewater tracer was also analyzed. Antibiotics and sucralose were measured by liquid chromatography-tandem mass spectrometry (LC-MS) after solid-phase extraction (SPE) developed by Brown (2020)[30].

For SPE, 1-L river samples were adjusted to pH 2 using hydrochloric acid 6N (Fisher Chemical) and two samples were spiked with 100 ng of deuterated d6 sucralose (Cayman Chemical) as the internal standard to assess SPE recovery. Samples were filtered with 0.7 µm Hach's glass fiber filters followed by 0.45 µm Whatman cellulose acetate membrane filters. The hydrophilic-lipophilic-balance (HLB) cartridges (SupelCo, bed wt. 200 mg and volume 6 mL) were sequentially preconditioned with 5 mL of LC-MS grade methanol (Fisher Chemical), and 5 mL of HPLC grade water (EMD Millipore). Samples were loaded onto HLB cartridges using tubing adaptors and Resprep 12-port SPE manifold (Restek) at a rate of 3-5 mL/min; after which the cartridges were rinsed into disposable culture tubes (VWR) with 2.5 mL of LC-MS grade methanol followed by 2.5 mL of LC-MS grade acetonitrile (EMD Millipore). The resulting extract was concentrated using a water bath (OAHEAT™ Model 5085) and a gentle stream of ultra-high purity carrier-grade nitrogen (Roberts Oxygen Company) using N-EVAP™111 nitrogen evaporator system to an approximate volume lower than 1 mL. The extract then was brought to a final volume of 1 mL using mobile phase (LC-MS grade methanol: LC-MS grade acetonitrile, 50:50), transferred to autosampler vials (VWR), and stored at -20 °C until analysis.

Stock solutions of azithromycin, sulfamethoxazole, Sucralose, sucralose-d6, and trimethoprim were made at the concentrations of 1 g/L by dissolving an appropriate amount of standard (Sigma-Aldrich) in HPLC Grade Methanol. The stock solution of ciprofloxacin was prepared by dissolving standard in HPLC Grade Methanol with 0.1% hydrochloric acid 6N[31]. Standard solutions used in LC-MS calibration were prepared at concentrations 2, 5, 10, 20, 50, 100, 200, 500, 1000 µg /L by serial dilution using HPLC grade methanol/ HPLC grade water (50/20 v/v). Antibiotics and sucralose were quantified by Thermo Fisher Accela/Velos HPLC-MS Ion Trap with Diode Array Detector, Poroshell 120 EC-C18 (Agilent), and Xcalibur (v.3.1.66.10) software for data acquisition. Mobile phase A and B are 1% formic acid in HPLC grade water and acetonitrile, respectively. Antibiotic concentrations were reported as the average of duplicate samples. Details of the LCMS method such as retention time and ions can be found in Brown 2020[30]. The level of detection limit (LOD) of each antibiotic is presented in *Appendix*, Table A7.

5.3.4 SPATIAL AND STATISTICAL ANALYSIS

Spatial data exploration and analysis were conducted using Hot Spot analysis (Getis-Ord Gi*) to identify watersheds in the study area and its neighboring watersheds with statistically significant antibiotic pollution than other watersheds. Spatial Autocorrelation (Global Moran's I) was performed to determine whether the index score of antibiotics pollution sources is statistically clustered. Then, Getis-Ord Gi* was used to determine “hot spots” of watersheds with elevated antibiotic pollution. Global Moran's I and Getis-Ord Gi* inputs include final index score of antibiotic pollution as Input field, Zone of Indifference as conceptualization of spatial relationships, Euclidean Distance as Distance Method, Distance Band of 16 km. Distance band was selected based on sensitivity analysis Z-score and P-Values versus distance band (*Appendix*, Figure A9) and ensured each watershed interacts with a few neighbors. In addition, environmental degradation and manure application are expected within 15 km of CAFO[32]. Previous studies reported effluent-origin ARGs was persistent up to 6.8km[33] and 20 km [34] downstream of discharge sites. Hot Spots analysis and Global Moran's I were carried out in ArcGIS 10.7.1. Lastly, counties with higher antibiotic pollution and their socioeconomic status (SES) were investigated by comparing the results of Hot Spot Analysis to environmental justice metrics such as median household income, percent in poverty, percent adults with less than high school diploma, percent non-Hispanic Black or African American, and non-Hispanic White. Data on environmental justice metrics were obtained from 2020 census data[35].

Antibiotic concentrations at modeled impacted sites were statistically compared to the reference sites representing lower human impact in the selected watersheds to evaluate whether sites that are modeled to be impacted by antibiotic pollution experience elevated antibiotic concentrations in the surface water. Welch's t-test has been used to explore the potential differences between antibiotic concentrations in modeled impacted sites and reference sites. Welch's t-test has been performed in MATLAB R2020b.

5.4 RESULTS AND DISCUSSIONS

5.4.1 CONTRIBUTION OF HOSPITALS TO MUNICIPAL WASTEWATER LOADING

We evaluated the loading contributions of hospital sewer discharges to receiving WWTP to better understand the potential influence of hospital antibiotic and AR loading on the municipal sewage system. Hospital sewer flows were estimated for174 hospitals across North Carolina. Those with higher capacity and thus higher sewage flow are located in urban areas such as Charlotte, Raleigh, Durham, and Winston Salem. The median and 90th percentile of hospital sewage flow are 12,934 and 54,601 gallons per day, respectively. After the connection between the hospitals and receiving WWTP was made, the aggregated hospital sewage flow received by a given WWTP was divided by WWTP design capacity to quantify the magnitude of hospital loads into sewer network. Our results indicate that approximately 40% of WWTPs (*n=112 out of 276*) receive and treat hospital sewage. In regard to the magnitude of contribution, more than 91% WWTPs with hospital sewage input *(n=103 out of 112)* receive less than 2% hospital sewage compared to WWTP capacity. WWTPs with hospital sewage input more than 2% *(n=9)* are discharging into large streams with higher instream flow and dilution capacity *(median of dilution factor of receiving streams=148.3*). Therefore, we do not expect that hospital sewage significantly influences antibiotic and antibiotic resistance downstream of WWTPs with hospital sewage compared to WWTPs without hospital sewage.

5.4.2 TOP THREE MOST IMPACTED WATERSHEDS BY ANTIBIOTIC POLLUTION ACROSS NORTH CAROLINA

Antibiotic pollution index was calculated for 360 HUC-10 watersheds across NC and ranked watersheds from most to least impacted by multiple point and nonpoint antibiotic pollution (i.e., WWTPs and CAFOs). A hospital metric was not included as an individual metric in the source index due to limited contributions to WWTP flows, as discussed in 3.1. Figure 11a displays the relative watershed ranking based on aggregated municipal wastewater as metric values of antibiotic point pollution in NC. Watersheds with elevated levels of antibiotic point pollution are located in urban regions with larger populations in central and south-central NC such as Charlotte, Raleigh, Durham, and

Asheville. These watersheds are relatively more impacted by antibiotic pollution discharged from WWTPs into surface waters. In addition, Figure 11b indicates that southeastern and east-central of the Coastal Plain region of North Carolina are at higher risk of antibiotic contamination with nonpoint pollution, especially from swine-producing facilities.

The top three most-impacted watersheds by antibiotic pollution are Sugar Creek in Catawba basin, Six Runs Creek, and Headwaters Northeast Cape Fear River which both are located in the Cape Fear basin. Sugar creek watershed (*HUC-12: 0305010301*) is located in Charlotte metropolitan area, the most populous city in NC[35]. Sugar creek is only exposed to point-source of antibiotics and receives the highest aggregated WWTP effluent in NC. Three major WWTPs are within the Sugar Creek watershed, which eventually feeds into the Catawba River Basin. These WWTPs are McAlpine WWTP which is the largest WWTP in NC[36], Irwin Creek WWTP, and Sugar Creek WWTP with a present design flow of 64, 15, and 20 million gallons per day (99.02, 23.21, 30.94 cubic feet second), respectively, and dilution factor of receiving stream (i.e. ratio of instream flow to present design flow) equal to 3.1, 2.9 and 2.4 based on mean annual flow, respectively[36, 37]. Downstream of these WWTPs are susceptible to the elevated level of antibiotics due to the high effluent flow rate combined with the relatively low dilution capacity of receiving streams.

Six Runs Creek (*HUC-12: 0303000605*) and Headwaters Northeast Cape Fear River watersheds (*HUC-12: 0303000702*) in Cape Fear Basin are significantly impacted by CAFOs. These watersheds are located in Duplin County and Sampson County which rank first and second swine-producing counties in the U.S.[18] with 4,452,768 cumulative number of allowable swine[29]. A total number of 287 swine-producing farms are located in Six Runs Creek and Headwaters Northeast Cape Fear River watersheds with cumulative allowable swine counts of 969,020 and 437,335, respectively, and total estimated antibiotic use equal to 80 and 40 tons of antibiotics per year. Two minor WWTPs are located in the Six Runs Creek watershed, Warsaw WWTP and Magnolia WWTP with a present design flow of 0.915 and 0.14 MGD (1.42 and 0.22 CFS), respectively. One WWTP is located in Headwaters Northeast Cape Fear River watersheds, MOUNT OLIVE WWTP with a present design flow of 1.66 MGD (2.57 CFS). Consequently, these watersheds are expected to be dominantly impacted by CAFOs.

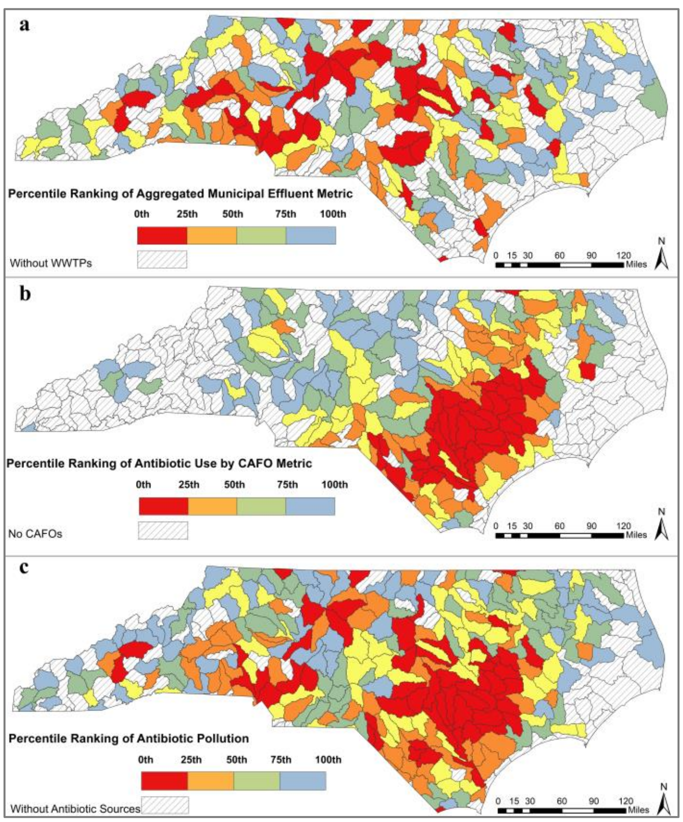

Figure 11. Geospatial distribution of antibiotic pollution a) aggregated WWTP effluent as point pollution metric, b) total antibiotic use by CAFOs as nonpoint pollution metric, and c) combined point and nonpoint pollution.

5.4.3 ANTIBIOTIC POLLUTION INDEX COMPARED AGAINST ENVIRONMENTAL JUSTICE METRICS

Antibiotic resistance is predicted to be the next pandemic[38]. Studies investigating racial and social disparities in antibiotic-resistant infections are vital in the prevention of pandemic inequality[38]. However, these studies are rare given the lack of racial and socioeconomic data in the health care system[38]. Some of the limited existing research suggests the disproportionate impact of antibiotic resistance on low-income communities and minorities, especially Black and Hispanics in the U.S.[39, 40] Results of a recent study highlighting environmental disparities of the disproportionate location of CAFOs in North Carolina[32] has led us to investigate the racial and socioeconomic status of counties that are modeled to be hot spots (i.e., statistically significant clusters) of antibiotic and AR sources. Figure 12 indicates hot spots of antibiotic pollution overlayed by NC Counties, where intense hot spots of antibiotic pollution are located in Duplin, Sampson, Greene, and Wayne counties. The average of environmental justice metrics for counties with hotspots of antibiotic pollution suggests that these counties have a high percentage of adults in poverty, low median household income, and a high percentage of adults with education below a high school diploma. For example, Duplin County and Greene County are the top 2 counties with the lowest education rate across NC (i.e., the highest percentage of adults with less than a high school diploma). In addition, the average percent of adults in poverty and non-Hispanic black among counties with hot spots of antibiotic pollution are 23.5% and 27.7%, respectively, while the median metrics for NC counties are 15.63% and 17.9%, respectively. The average of environmental justice indicators in counties with hot spots of antibiotic pollution alongside the median and 90th

percentile of the environmental justice indicators across NC counties can be found in *Appendix*, Table A8. Consequently, low-income and less educated communities in NC may be disproportionately impacted by antibiotic resistance, especially due to proximity to swine CAFOs.

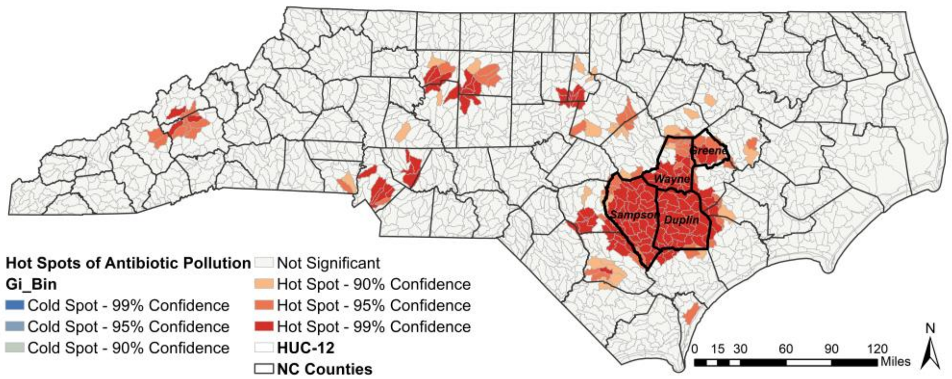

Figure 12. Hot Spots of antibiotic pollution, where the darkest hot spots have z-scores>2.58 and $p < .01$, intermediate hotspots have 1.97<z-score<2.57 and $p < .05$, and the lightest hot spots have 1.96<z-score<1.66 and $p < .1$. No Cold Spot was detected.

5.4.4 ENVIRONMENTAL CONCENTRATIONS OF ANTIBIOTICS IN MOST IMPACTED SITES

Four commonly detected antibiotics in surface water (azithromycin, ciprofloxacin, sulfamethoxazole, and trimethoprim) and sucralose as wastewater tracer were quantified for three impacted sites and one reference site chosen for lower pollution impact within each watershed that are modeled to be most impacted by antibiotic pollution (as shown in Figure 13). The mean concentration of all 4 antibiotic compounds downstream of WWTPs (i.e., Sugar Creek WWTP, Irwin Creek WWTP, and McAlpine Creek WWTP) was significantly higher than the mean antibiotic concentration in the respective reference site (REF1) in the urban watershed of Sugar creek ($P < .001$). In addition, sucralose has been detected at concentrations up to 25 times higher downstream of WWTPs than the upstream reference site.

Sucralose was not detected in any samples from Six Run Creek which is an agricultural watershed, indicating CAFO as the primary source of antibiotics in Six Run Creek (i.e., Stewarts Creek, Crane Creek, and Six Runs Creek). The mean of antibiotic concentrations in CAFO-impacted sites was significantly higher than the respective reference site (REF2) in Six Run Creek (p = .017). In the Northeast Cape Fear watershed, cumulative impacts of CAFOs and WWTPs on antibiotic concentrations can be observed in two sampling sites (i.e., NE Cape Fear 1 and 2). The highest concentrations of azithromycin, ciprofloxacin, sulfamethoxazole was noted in the NE Cape Fear River 2 downstream of WWTP and CAFOs, compared to the rest of the impacted sites. In addition, the mean of antibiotic concentrations in impacted (Panther, and NE Cape Fear 1 and 2) sites was significantly higher than the reference site (REF3) in the Headwaters NE Cape Fear River (p =.014). Azithromycin, ciprofloxacin, and sulfamethoxazole were persistent in all the collected samples collected from impacted sites. In addition, azithromycin was the predominant antibiotic among the four examined antibiotics, accounting for 54% of the total antibiotic concentrations in the impacted sites (Figure APX-2). By contrast, trimethoprim was only detected downstream of WWTP and not in agricultural sites *(n=4 out of 9).* The overall results indicate that antibiotic concentrations in modeled impacted sites are significantly higher than reference sites (p < .001).

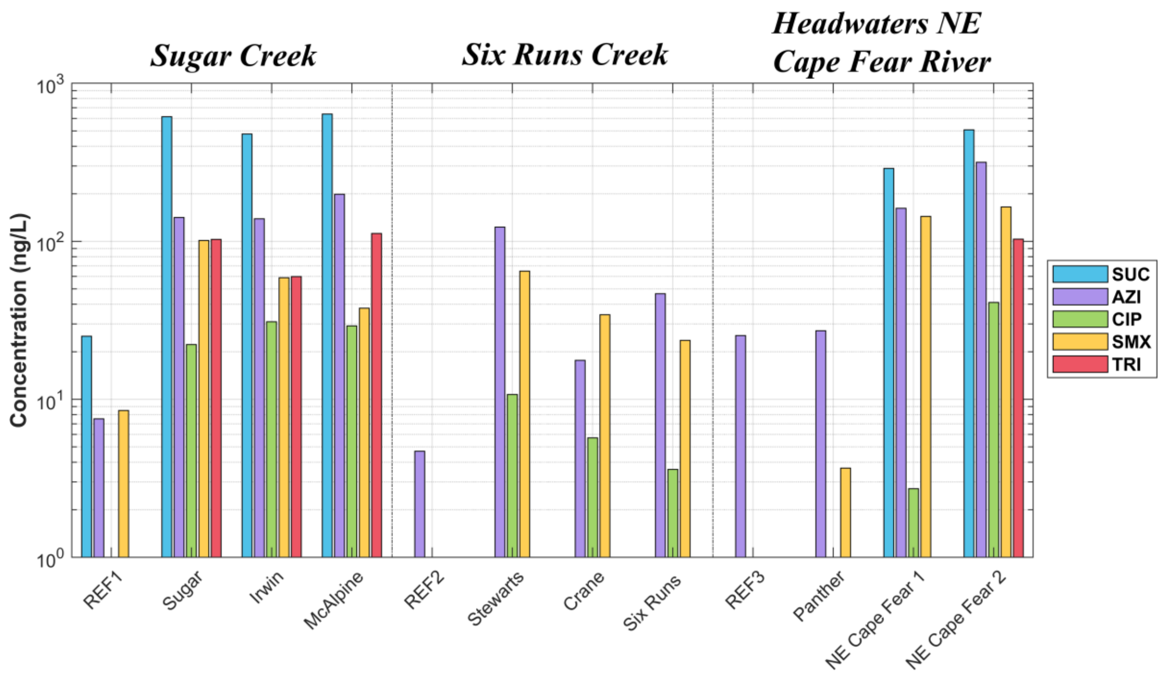

Figure 13. Measured antibiotic concentrations and sucralose concentrations in modeled impacted sites and respective reference sites within the top three most impacted watersheds.

5.5 CONCLUSIONS

Prior work detailing the occurrence of antibiotics within North Carolina highlights the significance of this study. Lambirth et al (2018) observed concentrations of taxa and antibiotic resistance genes in two wastewater treatment plants influents and effluents, upstream and downstream sites within Charlotte, NC [28]. This prior work paired with other potential sources of antibiotics besides WWTPs warrants further investigation of antibiotic compounds and resistance at the state level. In this study, we used environmental modeling to inform site selection for the assessment of antibiotic concentrations in modeled impacted watersheds associated with antibiotic point and nonpoint pollution. Significant differences were observed between antibiotic concentrations in modeled impacted sites and their respective reference sites ($p < .001$), highlighting the effectiveness of the developed model. The results of this work can provide quantitative information to policy analysts and decision-makers regarding the occurrence of antibiotics within North Carolina surface water. In addition, this study shows that the developed index of antibiotic pollution can facilitate site selection for the quantification of antibiotics, which can be applied to different states and regions.

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5.7 APPENDICES

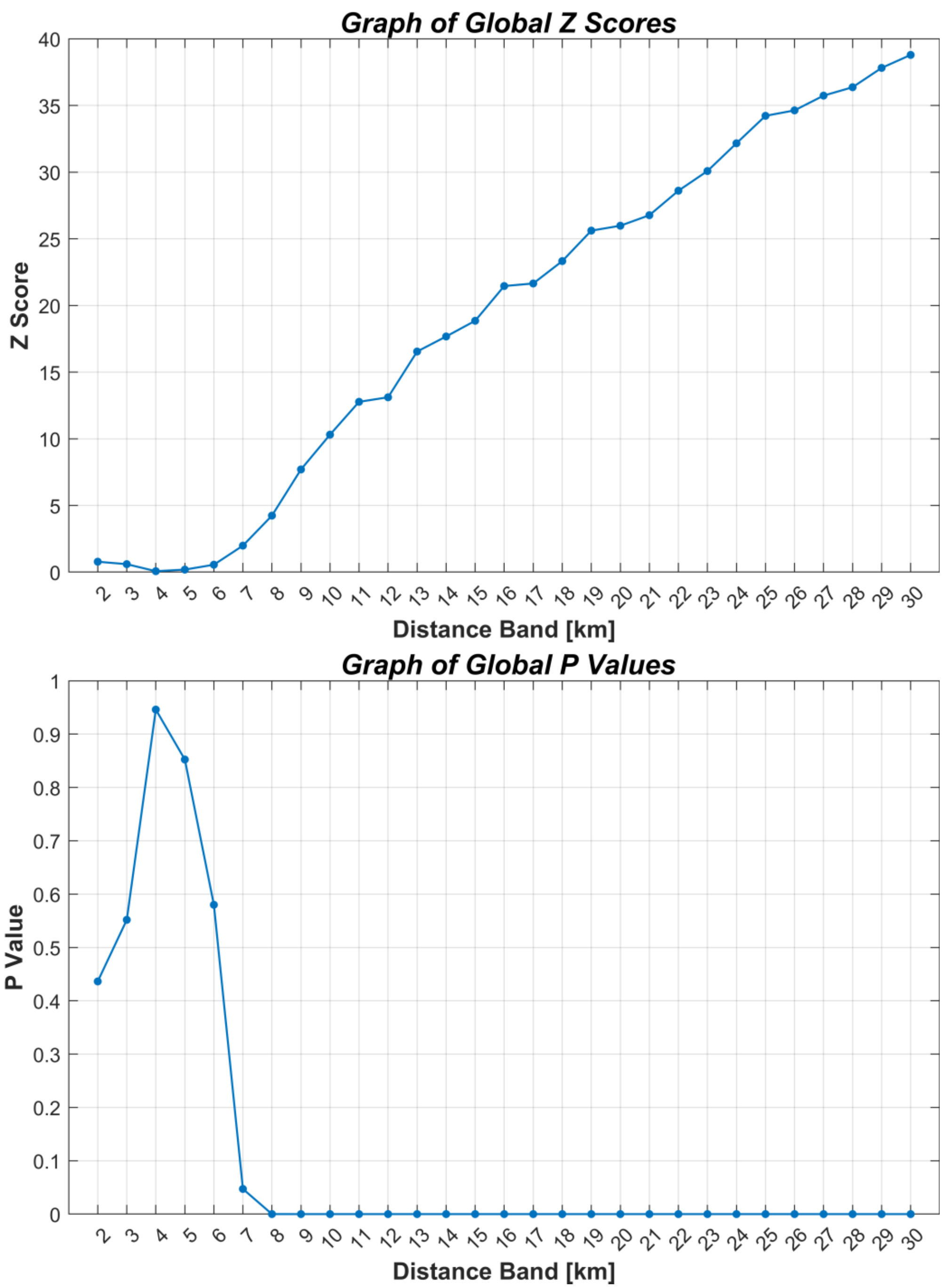

Figure A9. Sensitivity analysis of *p*-value and *z*-value from Global Moran's I analysis to selected distance band with input data of antibiotic pollution index in North Carolina.

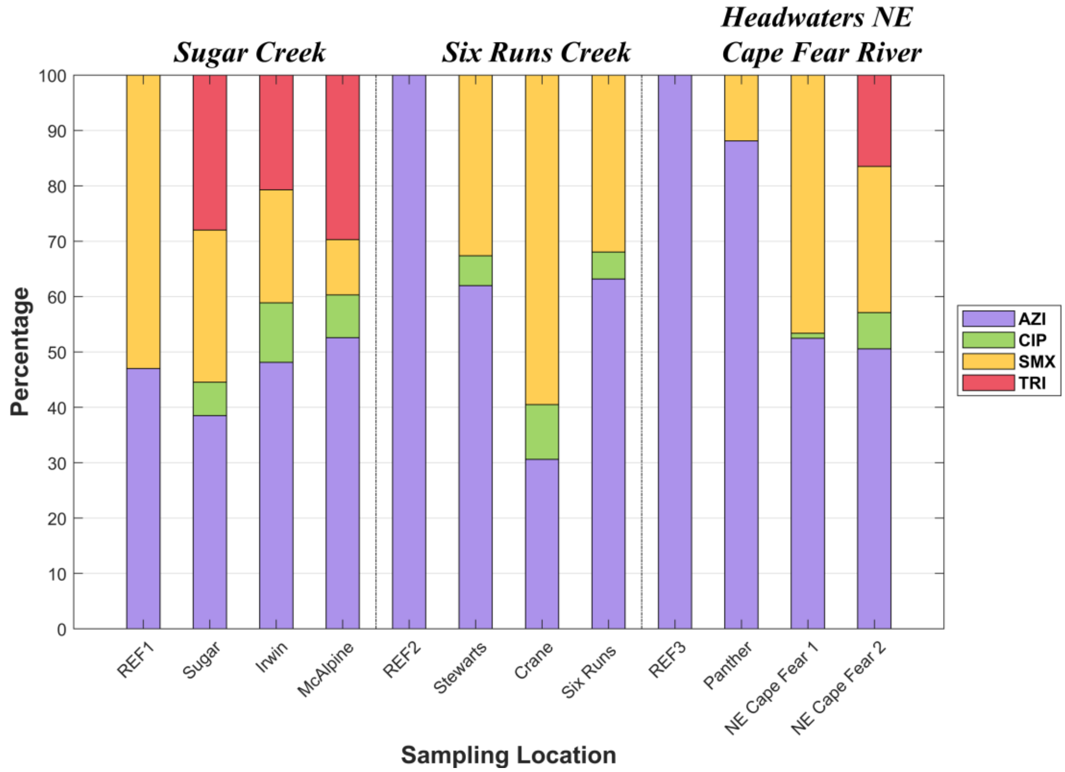

Figure A10. Measured environmental concentrations of selected antibiotics to the total antibiotic concentrations for each sampling site.

Table A6. Details of sampling locations. The geographic coordinate system is WGS 1984.

Label	X	Y	Watershed	HUC-10	Basin
Panther Creek	-77.8203	35.0412	Headwaters Northeast Cape Fear River	303000702	Cape Fear
Northeast Cape Fear River 1	-77.834	35.06553	Headwaters Northeast Cape Fear River	303000702	Cape Fear
Northeast Cape Fear River 2	-78.0177	35.19138	Headwaters Northeast Cape Fear River	303000702	Cape Fear
REF3	-77.8723	35.16873	Headwaters Northeast Cape Fear River	303000702	Cape Fear
Stewarts Creek	-78.2007	34.89098	Six Runs Creek	303000605	Cape Fear
Crane Creek	-78.2705	34.85441	Six Runs Creek	303000605	Cape Fear
Six Runs Creek	-78.231	35.04329	Six Runs Creek	303000605	Cape Fear
REF2	-78.228	34.9737	Six Runs Creek	303000605	Cape Fear
Sugar Creek	-80.8579	35.14191	Sugar Creek	305010301	Catawba
McAlpine Creek	-80.8802	35.06287	Sugar Creek	305010301	Catawba
Irwin Creek	-80.9141	35.19042	Sugar Creek	305010301	Catawba
REF1	-80.8925	35.20623	Sugar Creek	305010301	Catawba

Table A7. Details of retention times, ions, calibration curves, level of detection (LODs) are estimated as 3.3 S_y/S where Sy is the standard deviation of the response from the standard curve and S is the slope of the calibration curve. The method detection limit (MDL) is 2 ng/L.

Compound	**LOD (ng/L)**
Azithromycin	5.62
Ciprofloxacin	6.85
Sulfamethoxazole	4.56
Trimethoprim	9.51
Sucralose	29.01
Sucralose-d6	37.6

Table A8. The stations of environmental justice metric for impacted counties by antibiotics pollution; and median and 90th percentile across North Carolina's counties.

	% Adults with less than a high school diploma	***Median household income***	***Poverty status- % In poverty***	***% Non-Hispanic White***	***% Non-Hispanic Black***
Counties with hot spots of antibiotic pollution	24.02	$39,200	23.49	65.58	27.67
Median across NC	16.06	$45,078	15.63	73.39	17.90
90th Percentile across NC	22.31	$57,606	23.90	92.48	41.23

CHAPTER 6: SUMMARY, CONCLUSIONS, AND FUTURE WORK

6.1 SUMMARY OF FINDINGS AND CONCLUSIONS

The main goal of this research was to develop an integrative approach to identify and assess watershed vulnerability to contamination of antibiotics and antibiotic resistance (AR) and to use the developed approach to inform field study centered in North Carolina streams. This was accomplished through three studies; in the first study, we quantified antibiotic concentrations at WWTP discharge sites and identified streams more susceptible to antibiotic resistance across the U.S. In the second study, we assessed watershed vulnerability to antibiotic resistance occurrence by the development of the multimetric index that incorporates potential antibiotic point and nonpoint pollution, hydrologic conditions, and climate change. Lastly, we conducted a targeted field study quantifying selected antibiotics within three North Carolina watersheds that are modeled to be most impacted by potential antibiotic pollution (i.e., WWTPs and CAFOs).

We developed a spatial model using a Geographic Information System (GIS) that incorporates more than 13,000 WWTPs into the receiving streams across the U.S. Using the developed model, selected antibiotic concentrations were quantified at discharge sits, and streams at higher risks of antibiotic resistance presence were identified by comparing predicted antibiotic concentrations to antibiotic resistance safety threshold. Under mean annual flow conditions, one-third (*34.9%, n=4,629*) of sites surpassed the antibiotic resistance (ABR) safety threshold for ciprofloxacin, while under low instream flow conditions as 7-days 10-year low, and dilution factors in 76.9% (*n=9,885*) and 25.8% (*n=3,323*) of streams did not meet the ABR safety factor for ciprofloxacin and erythromycin, respectively.

Our results indicate that predicted no-effect concentrations (PNECs) for resistance selection exceedance is exacerbated for sites located on a smaller size stream defined by lower Strahler stream order below 4 (77.1% of sites). In addition, the impact of key parameters on model performance and PNECs exceedance were investigated. These parameters include varying instream flow conditions, varying effluent flow conditions, varying applied safety factors, and varying estimated environmental concentrations. The influence of streamflow is very pronounced, particularly for low instream flow conditions.

Watershed vulnerability to antibiotic resistance was assessed by the development of a multimetric index considering multiple antibiotic point and nonpoint pollution, hydrologic conditions, and climate change. In addition to WWTPs, other point sources (hospitals and total antibiotic prescription) and nonpoint sources (antibiotic use by food-producing animals and manure application) were incorporated into the model as well as dam storage ratio as the hydrologic indicator and projected minimum temperature change as climate change indicator. Our results indicate that midwestern watersheds are the most vulnerable to antibiotic resistance occurrences across the U.S. In addition, antibiotic nonpoint pollution was found to be the predominant factor impacting the antibiotic resistance vulnerability index among the majority of examined states (n= 40 out of 49).

The developed multimetric index was downscaled and adapted to identify the most impacted watershed by antibiotic pollution sources in North Carolina to be further investigated with field study. In addition, we used hot spots analysis to determine counties with intense hot spots of antibiotic-impacted watersheds and investigate the racial and socioeconomic status of identified counties. Our modeling results suggest that low-income and less educated communities are more impacted by potential risks associated with antibiotic and AR sources. In addition, antibiotic concentrations in modeled impacted sites were significantly higher than references, highlighting the ability of the developed model in sampling site selection.

6.2 LIMITATIONS

This study is limited to the accuracy and availability of datasets at the national level. Although significantly higher antibiotic concentrations are detected in the effluent of pharmaceutical manufacturing facilities3, 4, there is no nationwide dataset of geocoded manufacturing facilities, information regarding the presence of decentralized WWTPs or discharge to municipal WWTPs with quantitative attributes such as effluent flow. If such information becomes available, assessing the impact of pharmaceutical manufacturing effluent on the municipal wastewater system or/and impacts of industrial wastewater discharges containing antibiotics on surface water is suggested. In addition, antibiotic and ARG emissions from CAFOs into surface water were not quantified in this study. This is in part due to the absence of national CAFO datasets. CAFO datasets with coordinates, animal types, and allowable counts are available for a few states. USDA datasets of livestock and poultry inventory do not provide coordinates of animal farms, and the dataset is only available at the county level or watershed level not smaller than HUC-6. In addition, available datasets used in the study caused uncertainty due to the multiple timescales introduced within your analyses.

Different types of treatment methods implemented in WWTPs can reduce antibiotic and antibiotic resistance bacteria concentrations as well as ARG enrichment in treated effluent to various extents. The influence of the treatment level, on the other hand, is not fully accounted for in the model. Future studies require to incorporate treatment levels and their impact on antibiotic concentrations downstream of WWTPs. In addition, the dam storage ratio, which was used as a hydrologic condition indicator in the watershed vulnerability index, is a coarse estimate that does not take into account instream flow conditions, seasonal variations in streamflow, or other important hydrologic factors that influence antibiotic concentrations and spread of antibiotic resistance in the aquatic environment. Although a more thorough investigation of hydrologic conditions at the national level is challenging, future studies may add indications of receiving streamflow and seasonality. In addition, temporal variation of parameters impacting antibiotic pollution such as antibiotic consumption rates and effluent fluctuations have not been considered and molding analysis was performed based on steady-state assumption.

6.3 FUTURE WORK

Our results of the investigation of the racial and socioeconomic status of NC counties that are modeled to be most impacted by antibiotic pollution warrant a comprehensive study at the national level to ensure that low-income and minorities are not disproportionately affected by potential antibiotic resistance infection. This can party done by expanding the proposed sub-index of antibiotic point and nonpoint pollution to counties in the U.S. and comparing relative counties ranking against environmental justice metrics.

Despite the importance of CAFOs on the occurrences of antibiotics and antibiotic resistance in the environment, a nationwide geocoded dataset of CAFOs is not available. One project can aim to create a national database of CAFOs by integrating remote sensing into GIS. Once the dataset is available, a predictive model can be developed to quantify antibiotic concentrations in surface water emissions from CAFOs across the U.S. Antibiotics exerted from CAFOs can be calculated by total antibiotic use data provided by FDA[237] coupled with antibiotic exertion rate by the animal from literature[121]. Antibiotic concentration A geospatial model can incorporate antibiotic emissions from CAFOs into the agricultural runoff and near waterways.

One study can aim to improve the predictive of developed watershed vulnerability index to antibiotic resistance occurrences. Regression analysis between the outcomes of the developed resistance index and field study would be performed to estimate the optimal weighting of metrics and subindices. For this purpose, one potential monitoring dataset is the national monitoring dataset of antimicrobial resistance genes from 2,112 sites across the U.S., which was conducted in 2018-2019 by EPA as a part of the National Rivers and Streams Assessment (NRSA)[238]. As of today, however, this data set is not publicly available.

Another study can further the integrative field assessment performed in this work. ARG concentrations and antimicrobial communities would be quantified in modeled impacted sites identified in this study. In addition, antibiotic concentrations and antimicrobial communities in surface and groundwater can be assessed with a combined targeted and non-targeted approach in watersheds that are modeled to be most impacted by antibiotic pollution. In addition, the effect of streamflow characteristics on concentrations of antibiotics and ARG in the stream at the impacted sites can be investigated by collecting samples for at least 3 different hydrologic conditions including different times where low, average, or high flow is expected. In addition, although watershed vulnerability to antibiotic and AR contaminations was explored for North Carolina, we recommend applying and adapting the proposed approach to different locations and watershed levels to identify certain regions for prioritized watershed management.

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8. APPENDICES

8.1 MEASURED ENVIRONMENTAL CONCENTRATIONS OF AZITHROMYCIN AND TRIMETHOPRIM IN MUNICIPAL EFFLUENT.

Table A9. Literary values for measured environmental concentrations in treated wastewater effluent. a) azithromycin and b) trimethoprim.

A) Azithromycin

Reference	**Country**	**WWTP Info**	**Eff-Mean (ng/L)**
Panthia et al (2019)[239]	USA	Unknown	350.7
Gibs et al (2013)[221]	USA	Northwest Bergen County	40
Gibs et al (2013)[221]	USA	Ridgewood	85
Panditi et al (2013)[240]	USA	Miami-Dade North District	80
Nelson et al (2010)[225]	USA	Unknown- located in Los Angeles, CA	230.5
Loganathan et al (2009)[241]	USA	Murray WWTP	20.2
Haggard et al (2009)[242]	USA	Mud Creek WWTP	1003.3
Bartelt-Hunt et al (2009)[243]	USA	Omaha, NE	690.4
Bhandari et al (2008)[244]	USA	4 WWTP in the Midwestern U.S.	1110
		Median	**230.5**

B) Trimethoprim

Reference	**Country**	**WWTP Info**	**Eff-Mean (ng/L)**
Mohapatra et al (2016)[245]	USA	2 WWTP in the Southeastern U.S.	5543
Mohapatra et al (2016)[245]	USA	2 WWTP in the Southeastern U.S.	2969
Gibs et al (2013)[221]	USA	Northwest Bergen County	25
Gibs et al (2013)[221]	USA	Ridgewood	55
Panditi et al (2013)[240]	USA	Miami-Dade North District	98
Nelson et al (2010)[225]	USA	Unknown- located in Los Angeles, CA	59
Karthikeyan et al (2009)[223]	USA	7 WWTP in Wisconsin	390
		Median	**98**

8.2 DATA DIRECTORY

Table A10. Data Directory used in this study.

Data Set	Description	Data Source	Data Source Agency	Period	Methodology
WWTPs	Coordinates, Flow, Discharge Method, Level of Treatment	Clean Watersheds Needs Survey (CWNS) 2012[46]	U.S. EPA	January 2012 - December 2012	Survey
WWTPs	Coordinates	Permit Compliance System (PCS)[246]	U.S. EPA	Metadata Updated Date: March 2017	Survey
Mean Annual and Monthly Flow Estimates	Flow	National Hydrography Dataset Plus (NHDPlus) V2[247]	USGS and U.S. EPA	CONUS EROM: 1971 to 2000	Enhanced Unit Runoff Method (EROM) Gage-Adjusted Flow Estimates
The 7Q10 Flow Estimates	Flow	iSTREEM V2.1 [190]	American Cleaning Institute (ACI)	CONUS EROM: 1971 to 2000	7Q10 Flows at Gages: The Basis For the iSTREEM 7Q10 Flows NHDPlus-Based Routing Method
Stream Gage	Gage Location and Flow Statistics	National Hydrography Dataset Plus (NHDPlus) V2 [247]	USGS and U.S. EPA	Real-Time Data	-
Hospital	Number of Beds Personnel, and Coordinates	Data.Gov[248]	Homeland Infrastructure Foundation-Level Data (HIFLD)	Data set is reported in different time throughput 2012 to 2020	Survey
Measured Environmental Concentration (MECs)	MECs for Ciprofloxacin, Erythromycin and Sulfamethoxazole	Literature Review (*Appendix* Table A1) [32, 33]	-	Updated as Needed	-
Predicted No Effect Concentrations for Resistance Selection	PNECs for Ciprofloxacin, Erythromycin and Sulfamethoxazole	Bengtsson-Palme and Larsson (2016)[249]	-	2016	Modeling Approach Based on Minimal Inhibitory Concentrations (MICs)

NC Detailed Streets	Road Network Shapefile	ArcGIS Streetmap North America [250]	USGS	2020	-
WWTP Elevation	Elevation Layer	Seamless 1/3 Arc-Second Digital Elevation Model (Dem)[251]	USGS	2017	-
Watershed Boundary Dataset (WBD)	Hydrologic Unit Map	National Hydrography Dataset Plus (NHDPlus) V2[247]	USGS	2012	-
Animal Inventory	Type and Number of Livestock (chicken, swine, and cattle)	2017 Census of Agriculture [252]	United States Department of Agriculture	2017	-
Manure treated area	Area per watershed (Acres)	2017 Census of Agriculture [252]	United States Department of Agriculture	2017	-
Antimicrobials sold or distributed in 2019 for use in food-producing animals	Total antibiotic use by animals	Fda.gov[14]	Food and Drug Administration	2018-2019	
Antibiotic prescription Pattern	Antibiotic Prescriptions per 1,000 Persons by State	Outpatient Antibiotic Prescriptions - the United States, 2019 [253]	Centers for Disease Control and Prevention	2019	-
Dams	Dam Locations and Volume of Impounded Water	National Inventory of Dam [254]	U.S. Army Corps of Engineers	2018	-
Climate Change	Projected Minimum Temperature Chane (mean annual)	National Climate Change Viewer (NCCV)[208]	USGS	Baseline: 1950-2005 Projected: 2050-2074	20 downscaled CMIP5 climate models for the RCP8.5 emissions scenarios
Environmental justice metric	Non-Hispanic black, Non-Hispanic white, Percent living in poverty, Educational attainment	Non-Hispanic black[255]	U.S. Census Bureau	2020	