

Article

Occurrence of Antibiotic Resistant Bacteria in Urban Karst Groundwater Systems

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Abstract: Antibiotic resistance is a global concern for human, animal, and environmental health. Many studies have identified wastewater treatment plants and surface waters as major reservoirs of antibiotic resistant bacteria (ARB) and genes (ARGs). Yet their prevalence in urban karst groundwater systems remains largely unexplored. Considering the extent of karst groundwater use globally, and the growing urban areas in these regions, there is an urgent need to understand antibiotic resistance in karst systems to protect source water and human health. This study evaluated the prevalence of ARGs associated with resistance phenotypes at 10 urban karst features in Bowling Green, Kentucky weekly for 46 weeks. To expand the understanding of prevalence in urban karst, a spot sampling of 45 sites in the Tampa Bay Metropolitan area, Florida was also conducted. Specifically, this study considered tetracycline and extended spectrum beta-lactamase (ESBLs) producing, including third generation cephalosporin, resistant *E. coli*, and tetracycline and macrolide resistant *Enterococcus* spp. across the 443 Kentucky and 45 Florida samples. A consistent prevalence of clinically relevant and urban associated ARGs were found throughout the urban karst systems, regardless of varying urban development, karst geology, climate, or landuse. These findings indicate urban karst groundwater as a reservoir for antibiotic resistance, potentially threatening human health.



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Keywords: antibiotic resistant bacteria; antibiotic resistant genes; resistance phenotypes; urban karst groundwater

1. Introduction

Antibiotic resistance in bacteria has been recognized as a critical global concern for human and environmental health by the World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC) [1,2]. The presence of antibiotic resistant bacteria (ARB) in the environment is increasingly resulting in developed resistance to traditional antibiotics within humans [2–5]. One study found that over 70% of pathogenic bacteria are now resistant to at least one antibiotic, and over 2 million infections and 23,000 deaths in the United States can be attributed to antibiotic-resistant bacterial infections annually [6]. Although the overall cost of treating such infections and consequential loss of productivity is difficult to quantify, in the United States it is estimated to be \$20 billion in economic losses and \$35 billion in losses of productivity per year [6,7].

ARB occur naturally, but urban practices and development are accelerating their proliferation and dissemination in the natural environment, particularly in water resources [8]. Water resources are considered a viable pathway for transferring ARB and antibiotic

resistant genes (ARGs) to humans and animals [9,10] and are influenced by urban inputs, including urbanization, wastewater treatment plants (WWTP), hospital wastewater, stormwater runoff, septic tanks, leaking sewage lines, and pharmaceutical manufacturing effluents [11–25]. Agricultural practices also contribute to antibiotic resistance in the environment [26]; however, urban-related resistance is a greater threat to public health through multi-antibiotic and clinically relevant resistance [27–29]. Antibiotic resistance has been documented globally in surface water [30,31] and groundwater [31], where ARGs are shared through horizontal gene transfer mechanisms of conjugation, transduction, or transformation [32]. The development and dissemination of resistance throughout water environments threatens public health through a myriad of exposure pathways, such as treated drinking water and ambient water [30,33]; however, little to no actions have been taken to manage and regulate the threat in water resources [10,34].

Water-related antibiotic resistance studies have historically focused on WWTPs and surface waters. There is a lack of antibiotic resistance knowledge in groundwater resources, where the prevalence of antibiotics and resistance is just beginning to be documented [31]. Since 2.2 billion people worldwide rely on groundwater aquifers as drinking water sources, antibiotic resistance in groundwater can be of concern [30,31,35–38]. Moreover, groundwater systems connect a multitude of environments, including soil, surface streams, urban areas, and the subsurface, allowing for easy transport of pollutants from one source to another [36–46]. Groundwater also serves as a global reservoir for resistance influenced by urban practices [40,41,47] due to their natural intersections. Urban karst groundwater systems, where underground rivers and springs flow through urban areas riddled with caves and sinkholes that allow pollution to infiltrate rapidly, have also been sparsely studied. Nearly a quarter of the global population resides or relies on karst aquifers as drinking water sources [47,48]. Thus, it is pressing to address the prevalence of ARB in these systems as possible vectors negatively impacting human health [19,49–51]. Understanding the risk of antibiotic resistances to the environment and human health in urban karst groundwater settings is a primary gap in the current knowledge, specifically in the United States [23,31,35,36,45,52–56].

Some studies have detected antibiotics and antibiotic resistance in isolated or rural karst regions impacted by agricultural practices, including Lechuguilla Cave in New Mexico [57], Domica Cave in the Slovak Karst National Park [58], a rural karst aquifer in Northwest France [41], Parsik Cave located in a rural area of Turkey [59], and private wells in Midwest Ireland [60]. Resistance to beta-lactam, third and fourth generation cephalosporin [58], ampicillin, tetracycline, vancomycin, and other antibiotics [59] is prevalent in these karst settings; however, the interconnected nature of karst makes it difficult to determine the sources of resistance, such as human or animal, within karst areas due to mixed landuse [55,56,61]. Recent studies of karst systems in China suggest significant presence of antibiotics, such as macrolides, tetracycline, quinolones, and sulfonamides [37,55,62]. These antibiotics are primarily linked to urban inputs, including septic systems and WWTPs; however, these karst areas are primarily rural [54,62]. The presence of antibiotic compounds contributes to the development of ARB and ARGs, as seen in the study by Xiang et al. [55], where the antibiotic concentrations decreased downstream of the initial sampling site and the abundance of ARGs increased. Microbial source tracking markers for humans and food source animals are utilized to improve sourcing of resistance in these systems [55,60,61,63]. Furthermore, a greater prevalence of ARB has been suggested in karst waters than in surface waters associated with urban inputs [41,48,55,63,64].

The unique hydrogeology of karst systems, seasonality, and adjoining landuse impacts on the prevalence of ARGs associated with resistance phenotypes in urban karst aquifers is also not well elucidated [31]. Due to the increase of development in karst regions and urban pressures on karst groundwater resources, the purpose of this study is to demonstrate and understand the prevalence of ARB and associated ARGs within urban karst groundwater systems. To focus on the urban influence, tetracycline, macrolide, and beta-lactam (including third generation cephalosporin) antibiotics were targeted. These

antibiotics are extensively used and have been associated with both urban and clinical settings [65]. Furthermore, third and fourth generation cephalosporins targeted for ESBLs and macrolides are currently categorized as critically important antibiotics for medical use and tetracyclines are categorized as highly important antibiotics [66]. This study focuses on Bowling Green, Kentucky and the Tampa Bay Metropolitan Area, Florida as study areas representing a developing and well-developed urban karst groundwater system respectively, to analyze the proliferation of ARB in urban influenced systems. The study areas represent two types of karst regions (telogenetic and eogenetic, respectively), two different climate zones (temperate and subtropical, respectively), varying landuse impacts, a variety of karst features, varying recharge basin sizes, and vastly different population sizes to understand if ARB and associated ARGs are universally prevalent in urban karst systems. Due to the complex nature of karst hydrology and the interconnection of features, as well as the rapid movement of water and contaminants through the system, the prevalence of ARB and ARGs are analyzed spatially and temporally. The results from this study establish the foundation for understanding antibiotic resistance within urban karst groundwater systems.

2. Materials and Methods

2.1. Bowling Green, Kentucky Urban Karst

The urban metropolitan area of Bowling Green, located in Warren County, in the south-central region of Kentucky was selected as the primary study area for this research. Bowling Green is Kentucky's third largest city with approximately 74,363 residents [67]. The city is located within the Ohio Valley region, which experiences a temperate climate and variations in precipitation and temperature throughout the year. The monthly temperature trends seasonally, with the average high being 21 °C and the average low being 8 °C [68]. Precipitation occurs more frequently in the Fall and Winter seasons, with an average rainfall of 1310 mm annually and an average snowfall of 203 mm [68].

Classified as a karst landscape, Bowling Green does not have extensive soil development due to the lack of weathered material, since most bedrock is solutionally removed [47]. The soils in the area are typically 1–3 m thick and mainly consist of a variety of loams, clays comprising the Fredonia-Vertress-Urban and the Crider-Urban soil types [69]. The thin soils allow pollutants to be transported to the groundwater rapidly [70]. Bowling Green karst is classified as telogenetic, due to the soils covering the limestone, and is considered one of the largest cities to be built upon a karst sinkhole plain and an individual cave system [71]. The geology is comprised of fine-grain limestones, including St. Louis, Ste. Genevieve, and Girkin limestone formations, with the youngest layer being the Girkin, and the oldest St. Louis. There are two layers of chert, known as the Lost River Chert and Corydon Ball Chert, the latter of which is present at the contact between St. Louis and Ste. Genevieve formations [72]. Bowling Green lies within the Pennyroyal Sinkhole Plain, and much of it is developed atop the Lost River Chert bed, which does not erode as easily as the limestone, allowing the city to remain geologically stable for the most part [73]. The karst aquifer for Bowling Green is located in the Ste. Genevieve and St. Louis units.

The 10 sampling sites are comprised of a variety of karst features, including shallow epikarst wells, springs, cave streams, resurgences, and a primarily groundwater-fed surface stream (Figure 1). These features are encompassed within the city limits and fall within four of the seven main groundwater drainage basins [71].

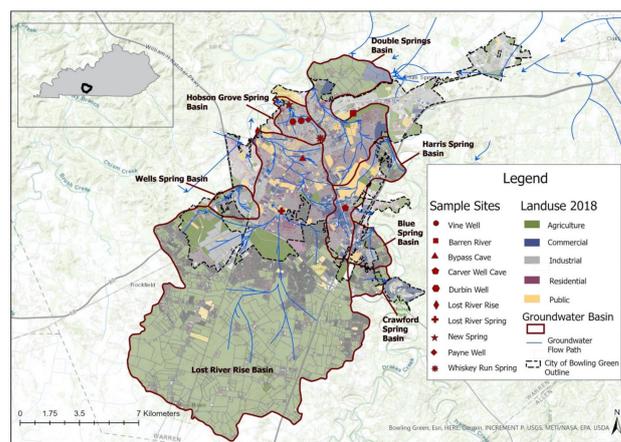


Figure 1. The sample sites, groundwater basins, groundwater flowpaths, and landuse for the Bowling Green, KY antibiotic resistance groundwater study area.

Bowling Green Landuse

Bowling Green, KY is a developing urban karst area and a mixture of residential, commercial, public, industrial, and agricultural landuses characterize the city (Figure 1). Landuse data was obtained from the City of Bowling Green Public Works department for 2018 and groundwater basin outlines from Western Kentucky University’s Center for Human GeoEnvironmental Studies.

2.2. Tampa Bay Metropolitan Area, Florida Urban Karst

To determine the prevalence of antibiotic resistance in urban karst groundwater systems, a developed urban karst area was also targeted. Spot sampling was conducted at 45 sites in the Tampa Bay Metropolitan Area, Florida. This area consists of four counties: Hernando, Pasco, Hillsborough, and Pinellas, all located in the west-central region of Florida. A fifth county, Citrus, was included within this study to incorporate groundwater watersheds that are assumed to be within the Tampa Bay Metropolitan Area, but resurge at features within Citrus County [74]. The population of the metropolitan area during this study period was 3,142,863 and the largest city within the area is Tampa with a population of 387,916 in 2019 [75]. The area climate is classified as subtropical and the average temperature of the area is 23 °C with an average rainfall of 117 cm [76].

This area is considered eogenetic karst due to the limestone being fairly young geologically and relatively exposed at the surface without having undergone extensive burial and induration. The geology is comprised of three aquifer systems, including the surficial, intermediate, and the Upper Floridan [77]. The surficial aquifer is comprised of Pliocene-Holocene unconsolidated siliciclastics, sand, and clay deposits. The intermediate aquifer is comprised of the Hawthorn Group and the Upper Floridan Aquifer is comprised of the Avon Park Formation and the Ocala-Suwannee Limestone Groups, which have contributed to the formation of the Floridan Aquifer, the primary karst groundwater aquifer for the state. The soils here are primarily sand and clay deposits ranging from thin (surface exposure) near the coast to 30–200 m thick inland and in the southern region of the area. The aquifer in this area, referred to as the Upper Floridan Aquifer, is extensively developed, ranging from 183 m to more than 425 m [77]. Unlike the Bowling Green study area, the karst features are primarily large springs (discharging millions of liters a day from the Upper Floridan Aquifer), sinks, and a groundwater-fed surface stream.

Tampa Bay Metropolitan Area, Florida Landuse

The landuse of the area is predominately commercial and residential along the coast, and rural landuse moving inland (Figure 2). Landuse data was obtained from the Florida Department of Environmental Protection Geospatial Open Data portal, and the Statewide

Land Use Land Cover layer was utilized. This area does not have well-defined groundwater basins and, as a result, the percentage of landuse within each basin was not determined.

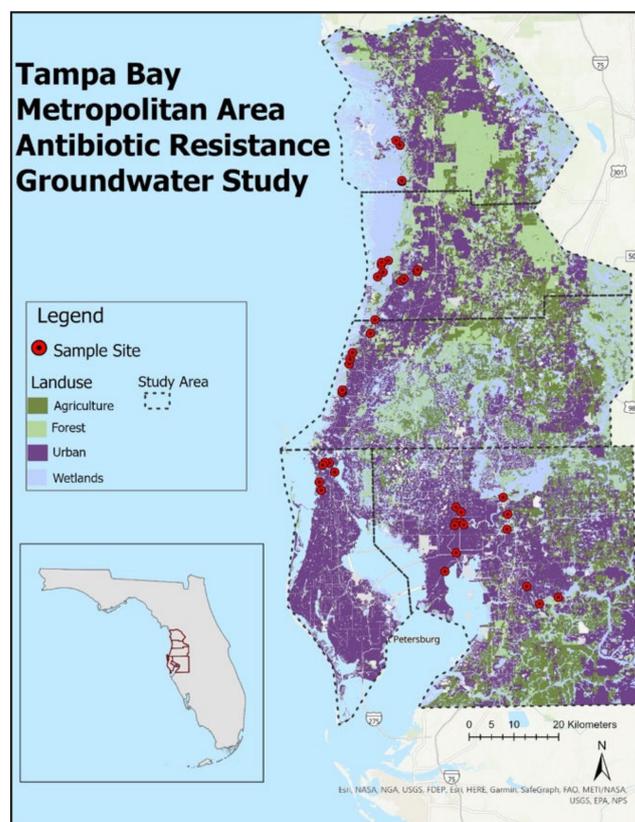


Figure 2. The sample sites and landuse for the Tampa Bay Metropolitan Area, FL antibiotic resistance groundwater study area.

2.3. Methodology

2.3.1. Sample Collection

Samples were collected at each of the Bowling Green, KY sites from 14 February, until 17 December 2018, at a weekly resolution to capture seasonal variation across the 46 weeks. The Vine Well site was only sampled for 29 weeks due to the well going dry [74]. Injection well features and Carver Well Cave were sampled using a well pump to extract the water samples. Spring features and Lost River Rise were sampled at the resurgence outlets directly. ByPass Cave was sampled from the cave stream located 10 m from the entrance. Water samples for the Barren River were collected along the edge of the riverbank.

Spot samples for the Tampa Bay Metropolitan, FL sites were collected from 3–9 January 2019 at the 45 sites. All sites were accessible at the surface and sampled directly. The groundwater-fed surface stream was sampled upstream and downstream of Tampa City and samples were collected along the edge of the riverbank. A liter of water was collected for each sample in a sterilized plastic Nalgene bottle and put on ice until refrigeration at 4 °C [74].

2.3.2. Sample Filtration

To prepare the water samples for ARB prevalence and ARG selection based on phenotypes, the maximum available volume of the collected sample from each site was filtered through two 0.45 µm filter papers (Advantec MFS, Inc., Tokyo, Japan) [74]. Between samples, the filtration unit was sterilized using 70% ethanol rinse, followed by a deionized water rinse. The apparatus was sterilized in an autoclave between weekly filtrations.

2.3.3. Plating Filtered Samples for Antibiotic Resistant Bacteria Prevalence and Resistance Phenotypes

Tetracycline and extended spectrum beta-lactamase (ESBLs) producing, including third generation cephalosporin, resistant *E. coli*, and tetracycline and macrolide resistant *Enterococcus* spp. were isolated for prevalence. These bacteria were selected because they are indicator bacteria and the antibiotics were selected due to common human use. Pre-enrichment cultures were prepared by adding the two 0.45 µm filter papers with the concentrated bacterial cells from water samples to 30 mL buffered peptone water (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Pre-enrichment broths were incubated at 25 °C for 2 h then at 42 °C for 6 h and then held at 4 °C until processed the next day. This step was used to resuscitate any bacteria damaged due to environmental stressors, including scarce nutrient conditions, transport, and the filtration process [74]. For *E. coli*, 0.5 mL of the pre-enriched culture was inoculated to (1) 2.5 mL of MacConkey broth (MCB, Becton, Dickinson and Company, Franklin Lakes, NJ, USA), (2) MCB supplemented with 4 mg/L of cefotaxime (third generation cephalosporin resistance) (MCB + CTX), and (3) MCB supplemented with 16 mg/L of tetracycline (MCB + TET), and all three plate types per sample were incubated at 42 °C for 18 h [26]. For *Enterococcus* spp., 0.5 mL of the pre-enrichment was transferred to (1) 2.5 mL of Enterococcosel broth (ECB, Becton, Dickinson and Company), (2) ECB supplemented with 8 mg/L of erythromycin (macrolide resistance; ECB + ERY), and (3) ECB supplemented with 16 mg/L of tetracycline (ECB + TET), and all three plate types per sample were incubated at 37 °C overnight. The enrichment step using antibiotic supplemented broth cultures was used to reduce background bacterial population by killing or inhibiting the growth of the susceptible bacteria while selecting for the resistant bacterial population [78]. This step increases the sensitivity of the assay to target the resistant population. Since the antibiotics were used at their minimum inhibitory concentrations (MIC), which kills or inhibits the susceptible population, coupled with a relatively short incubation period, the probability of bacteria becoming resistant through horizontal gene transfer or mutation is minimal [26,78].

Following incubation, MCB, MCB + CTX, and MCB + TET *E. coli* enrichments were swabbed onto (1) modified membrane thermotolerant *E. coli* media (mTEC, Becton, Dickinson and Company, Franklin Lakes, NJ, USA), (2) mTEC + CTX, and (3) mTEC + TET plates, respectively. All three plate types per sample were incubated at 37 °C for 18–24 h. ECB *Enterococcus* spp. enrichments were swabbed onto (1) Slanetz and Bartley Medium (SBM, Oxoid, Thermo Fisher Scientific, Inc., Waltham, MA, USA), (2) SBM + ERY, and (3) SBM + TET. All three plate types per sample were incubated at 35 °C for 4 h then at 44 °C for 48 h. The pre-enriched *E. coli* cultures were plated onto (1) CHROMagar™ ESBL Chromogenic Media (ESBL, Paris, France) to select for Gram-negative bacteria producing ESBLs, including third generation cephalosporins. For each of the selected ARB, up to two bacterial isolates were presumptively isolated based on characteristic appearance on the respective selective media. The isolates were inoculated in 1 mL of tryptic soy broth (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). After overnight incubation at 37 °C, isolates were preserved with 15% glycerol and stored at −80 °C. The isolates were stored in 100 µL aliquot at −80 °C until PCR confirmation [74]. All antibiotics were obtained from Millipore Sigma (St. Louis, MO, USA) and the Clinical Laboratories Standards Institute resistance breakpoint concentrations based on their MIC were used [79].

2.3.4. PCR Confirmation of Positive Antibiotic Resistant Bacterial Isolates

Since traditional plating techniques may not be as reliable, the selected antibiotic resistant bacteria isolates were confirmed through PCR analysis to ensure the correct bacteria (*E. coli* or *Enterococcus* spp.) were identified [80–82]. The saved isolates were first prepared for PCR through BAX lysis method for DNA isolation (Hygiena, CA, USA) using Eppendorf's epMotion 5075 (Eppendorf, Enfield, CT, USA). The instrument utilized 10 µL of the isolate stored in aliquot and 50 µL of BAX lysis buffer. The DNA samples were then prepared for PCR confirmation using primers for *E. coli* and *Enterococcus* spp. (Table S1).

AB 2720 Thermal Cyclers (Applied Biosystems, Beverly, MA, USA) were used in the PCR assay with standard cycles for *E. coli* and *Enterococcus* spp. The *Enterococcus* spp. isolates were confirmed using BioRad CFX 96 Real Time machine with the SYBR Green PCR assay qualitative endpoint PCR, excluding late amplifiers as false collected positives. The *E. coli* isolates were confirmed using gel electrophoresis. Once confirmed, the positive isolates were transferred to new master plates for further ARG analysis.

2.3.5. Antibiotic Resistant Gene Analysis for Resistance Phenotypes

The PCR confirmed positive resistant isolates were analyzed to determine the presence of known ARGs using end point PCR on gel electrophoresis for the targeted ARGs [83–94] (Table S1).

2.3.6. Data Handling

Landuse data utilized in this study were managed in Esri ArcGIS Pro software. The “summarize within” tool in ArcGIS Pro was used to determine the percentage of landuse within each of the seven groundwater basins for the Bowling Green study area. ARB and ARG detection frequency data were managed in OriginPro. ARG detection frequencies were calculated by the isolates positive for an ARG out of all positive resistant isolates for that antibiotic group, for each study area, for (1) bacteria type for overall detection frequency, (2) by site for spatial detection frequency, and (3) by site and meteorological season parameters as defined by the National Oceanic and Atmospheric administration (NOAA) [95]. For the Florida study area, spatial detection frequency was calculated by the total number of sites with detected prevalence for an ARG out of the 45 sites, due to the spot sampling approach for this study area.

3. Results and Discussion

3.1. Prevalence of Antibiotic Resistant Bacteria and Antibiotic Resistant Genes Associated with Resistance Phenotypes in Urban Karst Groundwater Systems

There is a ubiquitous presence of antibiotic-resistant *Enterococcus* spp. and *E. coli* indicator bacteria in Bowling Green, KY (Figure 3; Tables 1 and S2) and the Tampa Bay Metropolitan Area, FL (Figure 4; Tables 1 and S2). Tetracycline-resistant *E. coli* and *Enterococcus* spp. isolates are the most prevalent in both study areas, reflecting the extensive overuse of this antibiotic class and dissemination throughout the environment. Within this study, macrolide resistance in the *Enterococcus* spp. isolates is also extensively detected. The prevalence of beta-lactam resistance in both study areas is concerning because this antibiotic class is used sparingly to reduce the development of resistance and ensure usability to protect human health. These results suggest that antibiotic resistance is widespread and well-developed within the two urban karst groundwater study areas, being able to persist in differing karst environments and through seasonal changes. Variations in ARGs associated with resistance phenotypes both spatially and temporally indicate environmental factors influencing their development and presence; however, the high detection frequencies at most sample sites demonstrate the ability for urban karst groundwater systems to be vectors for ARG transmission and ARB development.

Tetracycline antibiotics and resistant genes are widespread in all ambient water environments, particularly groundwater [28] and karst groundwater [41,44,49,54,62–64]; however, there is limited research on tetracycline ARGs within urban karst groundwater systems. In Bowling Green, a high percentage of tetracycline resistant gene *tetM*, in *Enterococcus* spp. bacteria, is detected (87% of tetracycline resistant *Enterococcus* spp. isolates; Figure 3). This gene is mainly associated with municipal and hospital wastewater [30,84]. Although *tetM* is found in other environments, including agricultural and rural, a greater prevalence is detected in areas significantly impacted by urbanization [26]. Tetracycline-resistant gene *tetS* (1%; Figure 3), *tetO* (1%; Figure 3), and *tetL* (10%; Figure 3) are also detected in *Enterococcus* spp. isolates. These genes are considered clinically relevant and detected in wastewater [84]. Tetracycline-resistant genes *tetA* (55% of tetracycline resistant *E. coli* isolates; Figure 3) and

tetB (45%; Figure 3) in *E. coli* bacteria are detected, which are primarily associated with untreated sewage, wastewater, and drinking water [26,30,84,96,97]. Unlike the present study where a high detection of these genes is found within urbanized karst groundwater, a study by Stange and Tiehm [63] found low detection of *tetA* and *tetB* in a primarily forested and agricultural karst area in Germany. The study by Stange and Tiehm detected urban influence to the groundwater system, indicating a potential source of these genes, highlighting the interconnected nature of karst groundwater. Karst systems exhibit mixed landuse impacts to the groundwater; however, the findings in both studies indicate the influence of urbanization on ARGs.

Table 1. Prevalence of antibiotic resistance for all samples in the Bowling Green, KY and Tampa Bay Metropolitan Area, FL study areas.

Percent Positive of Antibiotic Resistant Samples					
		Percent Positive for the Bowling Green, KY Study Area Samples (%)	Number of Positive Samples for the Bowling Green, KY Study Area out of All Samples (<i>n</i> = 443)	Percent Positive for the Tampa Bay Metro Area, FL Study Area Samples (%)	Number of Positive Samples for the Tampa Bay Metro Area, FL Study Area out of All Samples (<i>n</i> = 45)
<i>Enterococcus</i> spp. Resistant Bacteria	Macrolide Antibiotic Resistance	58	256	13	6
	Tetracycline Antibiotic Resistance	93	412	40	18
<i>E. coli</i> Resistant Bacteria	Tetracycline Antibiotic Resistance	89	394	27	12
	Beta-Lactam (including 3rd generation cephalosporin) Antibiotic Resistance	65	286	18	8

Similar to the Kentucky study area, the tetracycline-resistant genes *tetM* (70.3% of tetracycline resistant *Enterococcus* spp. isolates), *tetL* (18.9%; Figure 4), *tetO* (5.4%; Figure 4), and *tetS* (5.4%; Figure 4) are detected in the *Enterococcus* spp. isolates from the Florida study area. The tetracycline-resistant genes, *tetA* and *tetB*, in the Florida *E. coli* isolates are also detected with a higher frequency of *tetB* (57.1% of tetracycline resistant *E. coli* isolates; Figure 4) than *tetA* (42.9%; Figure 4). This is unlike the Kentucky sites, where a slightly greater detection of *tetA* than *tetB* (Figure 3) is found. These two genes are primarily associated with urbanization [26,30,84,96,97] and the detection in both locations highlights this influence on the groundwater systems. The ranking of detection frequencies is the same between the two study areas for this antibiotic class, highlighting the influence of urbanization on the prevalence of ARGs associated with resistance phenotypes in urbanized karst regions.

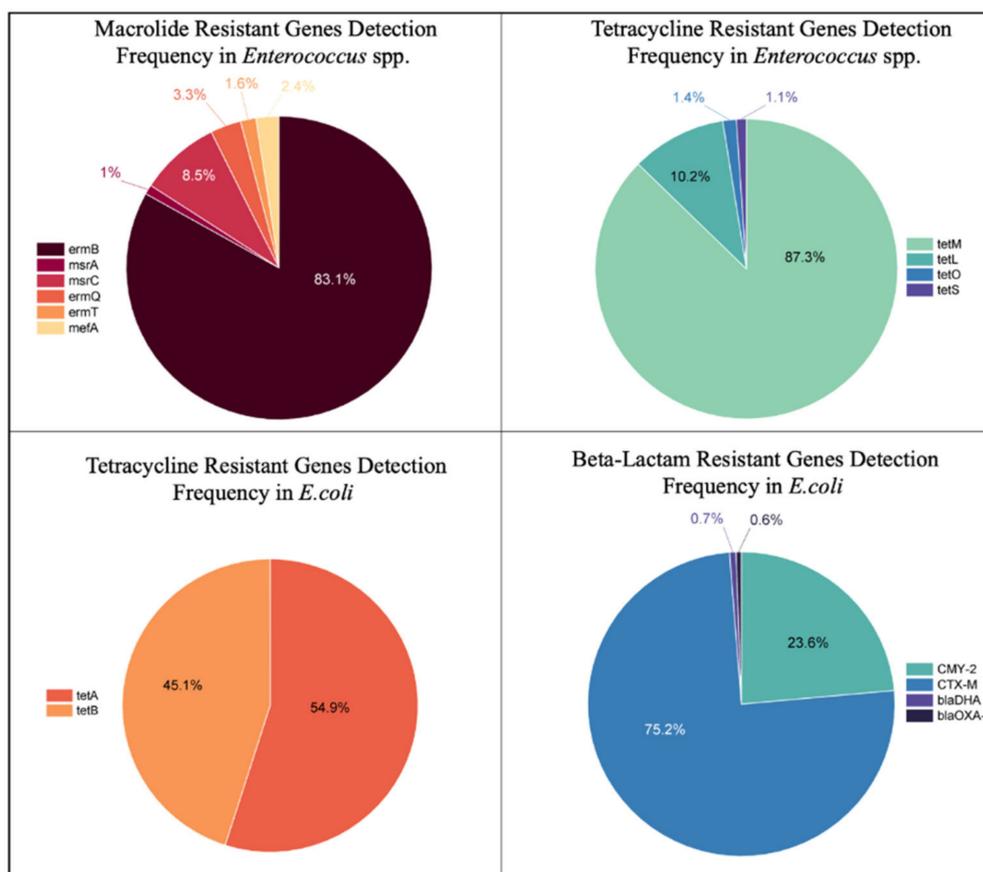


Figure 3. Detection frequencies of ARGs associated with resistance phenotypes for all *E. coli* and *Enterococcus* spp. isolates in the Bowling Green, KY urban karst groundwater system.

Macrolide antibiotics have received limited attention in karst groundwater, but have been detected in urban influenced karst systems [54,62]; however, macrolide ARGs are overlooked in karst systems. The macrolide-resistant gene *ermB* (81% of macrolide resistant *Enterococcus* spp. isolates; Figure 3), an ARG commonly sourced to wastewater effluent [30,84], is detected in the *Enterococcus* spp. bacteria within the Bowling Green study area. This gene was also detected in the study conducted by Stange and Tiehm; however, the prevalence was only captured in half of the samples [63] and not as ubiquitous as in this urbanized karst study. Macrolide resistance is also detected for the *mefA* (2%; Figure 3) and *msrA* (1%; Figure 3) genes [84], which are associated with municipal waste. The *msrC* (8%; Figure 3) [85], *ermT* (2%; Figure 3) [98], and *ermQ* (3%; Figure 3) [99] genes, primarily associated with agricultural waste, are also detected. The *ermB* gene is the only detected macrolide resistant gene in the *Enterococcus* spp. isolates in the Florida study area (100%; Figure 4).

Some studies have considered the prevalence of beta-lactam antibiotics and ARGs in karst groundwater; however, their focus was on rural karst [58] and there was low detection of beta-lactam ARGs [55,63]. The *E. coli* isolates resistant to beta-lactam antibiotics are captured in both study areas. The beta lactam-resistant genes in the Bowling Green *E. coli* bacteria exhibit the *bla*_{CTX-M} (76% of beta-lactam resistant *E. coli* isolates; Figure 3) and *bla*_{CMY-2} (23%; Figure 3) genes, however, the Florida samples exhibit a higher detection of *bla*_{CMY-2} (58.8%; Figure 4) than *bla*_{CTX-M} (29.4%; Figure 4). The *bla*_{CTX-M} and *bla*_{CMY-2} genes are the primary beta-lactam genes detected in both systems, which are commonly found to be discharged from WWTPs [26]. The ESBLs resistance phenotype is primarily the *bla*_{CTX-M} gene, and the other beta-lactam ARGs are detected in the third generation cephalosporin resistance phenotype (*bla*_{CTX-M}, *bla*_{CMY-2}, *bla*_{DHA}, *bla*_{OXA-1}). Beta-lactam resistance in Bowling Green is also detected for the *bla*_{OXA-1} (1%; Figure 3) [84] and the *bla*_{DHA} (1%; Figure 3) [97]

genes, which are also mainly associated with municipal wastewater. There is no detection of the *bla*_{DHA} gene in the Florida spot samples, but a higher detection of the *bla*_{OXA-1} (11.8%; Figure 4) gene than in the Kentucky samples (0.6%; Figure 3). Our findings are similar to the study by Xiang et al. [55], which exhibited an urban influence from WWTPs and septic tanks, detecting a low frequency of the *bla*_{OXA-1} gene. This gene is urban related [84] and the higher detection frequency in a larger urban area may reflect the influence of greater urbanization impacts on the sample sites.

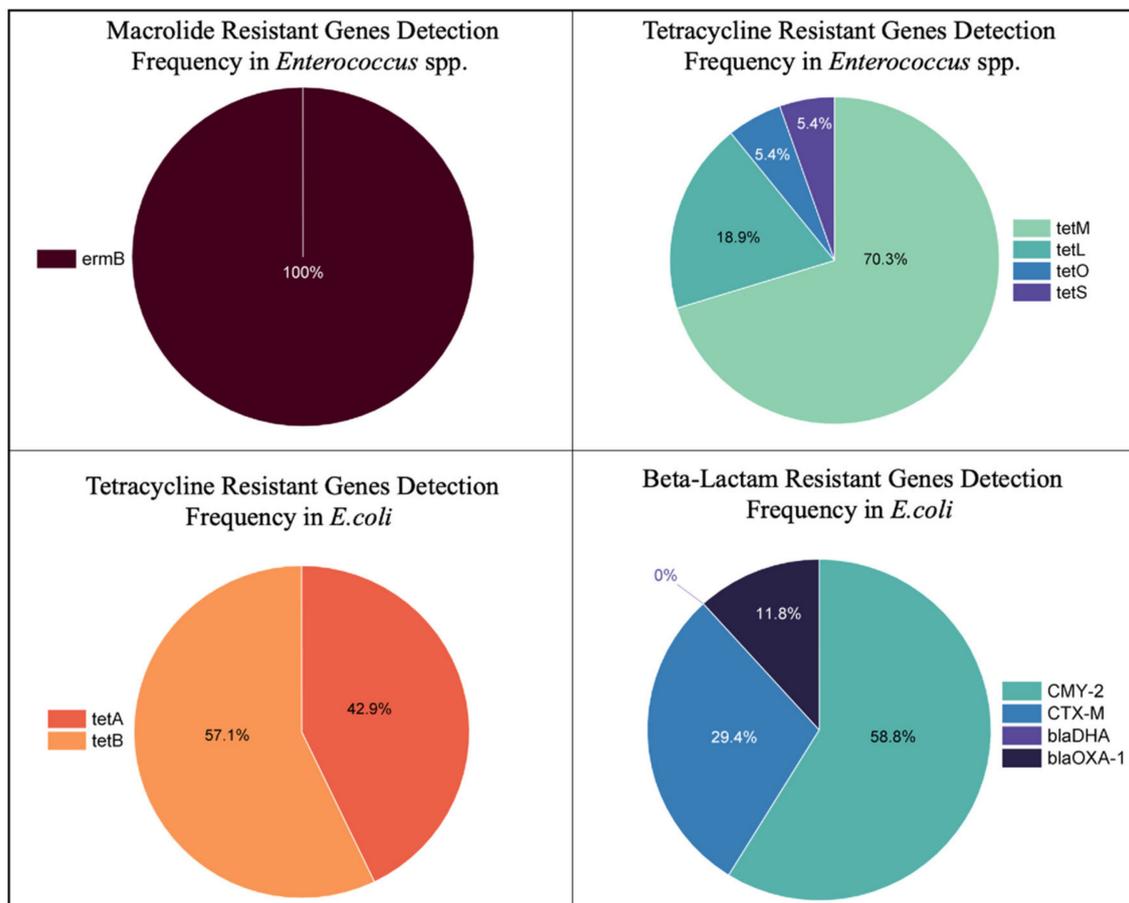


Figure 4. Detection frequency of ARGs associated with resistance phenotypes for all *E. coli* and *Enterococcus* spp. isolates in the Tampa Bay metropolitan Area, FL urban karst groundwater system.

The ARGs detected in this study are primarily associated with urban practices and clinically relevant antibiotic classes, but have been detected in rural and agricultural sources, indicating the vast dissemination of resistance within the environment, particularly water resources. The extensive detection frequencies of these ARGs in both study areas, as well as the influence of urban landuse, supports the concept of urbanization increasing the prevalence of ARGs associated with resistance phenotypes in karst groundwater resources [26,31,37,44,49,55,63,64,100].

3.2. Spatial Trends of Antibiotic Resistant Gene Association with Resistance Phenotypes and Landuse for Bowling Green, KY

The samples sites are primarily influenced by residential landuse within the city limits; however, the Lost River Rise Basin has a high percentage of agricultural landuse contribution outside of the city limits, potentially influencing ARG prevalence (Table 2). The total detection frequency of the targeted ARGs at each sample site is also provided to

understand the spatial distribution of ARGs within the urban karst groundwater system (Table 3).

Table 2. Landuse percentage and sample site location within the defined groundwater basins for in Bowling Green, KY.

Basin	Sites	Agricultural (%)	Commercial (%)	Residential (%)	Public (%)	Industrial (%)
Harris Spring Basin	Carver Well Cave	15	15	27	19	4
Lost River Rise Basin	Lost River Spring, ByPass Cave, Lost River Rise	62	2	19	4	4
Hobson Grove Spring Basin	New Spring, Durbin Well, Vine Well, Payne Well, Whiskey Run Spring	7	6	41	16	7
Double Springs Basin	Barren River	32	16	27	11	2

3.2.1. Bowling Green, KY Detection of Antibiotic Resistant Genes Associated with Resistance Phenotypes for *Enterococcus* spp.

The detection frequency of *Enterococcus* spp. isolates resistant to macrolides in relation to landuse is spatially depicted in Figure 5 and detailed in Table 3. All sample sites exhibit over 60% detection frequency by site for *ermB* and less than 12% for *msrC*, *msrA*, *ermQ*, *ermT*, and *mefA* (Table 3). The *ermB* gene is detected at all sample sites with the highest detection at Carver Well Cave (93%; Table 3) and the lowest at Payne Well (61%; Table 3), which are both primarily influenced by residential landuse (Table 2). The *msrC* gene is detected at all the sample sites except for Carver Well Cave. The *msrA* gene is only detected at the ByPass Cave (3%; Table 3), Carver Well Cave (6%; Table 3), and New Spring (2%; Table 3) sites, all primarily influenced by residential landuse (Table 2). The *ermQ* gene is detected at the ByPass Cave (10%; Table 3), Durbin Well (5%; Table 3), New Spring (4%; Table 3), Whiskey Run Spring (4%; Table 3), Payne Well (6%; Table 3), and Vine Well (3%; Table 3) sample sites, all of which, except for ByPass, are primarily influenced by residential landuse (41%; Table 2). The ByPass Cave site falls within the Lost River Rise Basin, which is more influenced by agricultural landuse (62%; Table 2); however, the ByPass sample site is directly impacted by commercial and residential landuse, potentially introducing undiluted urban inputs (Figure 1). The *ermT* gene is only detected at the Lost River Rise (3%; Table 3), Lost River Spring (4%; Table 3), and Payne Well (11%; Table 3), indicating potential agricultural and urbanization influence (Table 2). The *mefA* gene is detected at the Durbin Well (3%; Table 3), Whiskey Run Spring (8%; Table 3), Lost River Spring (2%; Table 3), Payne Well (11%; Table 3), and Vine Well (3%; Table 3) sample sites, all of which, except for Lost River Spring, are primarily influenced by residential landuse (41%; Table 2). The Payne Well site exhibits the highest detection frequency for *ermT* and *mefA* (11%; Table 3) and the second highest detection frequency for *msrC* (11%; Table 3) and *ermQ* (6%; Table 3), following Whiskey Run Spring (12%; Table 3) and ByPass Cave (10%; Table 3), respectively. The sample sites are directly impacted primarily by residential landuse; however, the interconnected nature of the karst groundwater system within the groundwater basins exposes agricultural landuse impacts to the sample sites, specifically for the Lost River Spring, Lost River Rise, and Barren River sites, as highlighted by the greater detection of agricultural related ARGs (*msrC*, *ermT*, *ermQ*) at these sites. Regardless of mixed landuse, the urban associated gene, *ermB*, exhibits a consistent prevalence.

Table 3. Total detection frequencies of ARGs for resistance phenotypes of *Enterococcus* spp. and *E. coli* isolates.

Bowling Green, KY Antibiotic Resistant Gene Detection Frequency (%)													
Sample Site	Total Number of Isolates (n)	Macrolide Resistant Genes											
		<i>ermB</i> (%)	Number of Isolates (n)	<i>msrA</i> (%)	Number of Isolates (n)	<i>msrC</i> (%)	Number of Isolates (n)	<i>ermQ</i> (%)	Number of Isolates (n)	<i>ermT</i> (%)	Number of Isolates (n)	<i>mefA</i> (%)	Number of Isolates (n)
ByPass Cave	58	76	45	3	2	8	5	10	6	0	0	0	0
Durbin Well	58	85	50	0	0	5	3	5	3	0	0	4	2
Barren River	53	87	48	0	0	9	5	0	0	0	0	0	0
Carver Well Cave	33	94	31	6	2	0	0	0	0	0	0	0	0
Lost River Rise	63	83	54	0	0	11	7	0	0	3	2	0	0
New Spring	54	87	48	2	1	5	3	4	2	0	0	0	0
Whiskey Run Spring	50	75	38	0	0	12	6	4	2	0	0	8	4
Lost River Spring	51	84	43	0	0	10	5	0	0	4	2	2	1
Payne Well	36	61	22	0	0	11	4	6	2	11	4	11	4
Vine Well	36	77	30	0	0	10	4	3	1	0	0	3	1
Tetracycline Resistant Genes													
Sample Site	Total Number of Isolates (n)	<i>tetM</i> (%)	Number of Isolates (n)	<i>tetL</i> (%)	Number of Isolates (n)	<i>tetO</i> (%)	Number of Isolates (n)	<i>tetS</i> (%)	Number of Isolates (n)				
ByPass Cave	89	90	80	9	8	1	1	0	0				
Durbin Well	90	84	76	12	11	3	3	0	0				
Barren River	89	87	77	8	7	1	1	3	3				

ARG associated with resistance phenotypes for *Enterococcus* spp.

Table 3. Cont.

	Carver Well Cave	83	84	70	12	10	0	0	2	2					
	Lost River Rise	88	82	72	14	12	3	3	1	1					
	New Spring	86	86	74	13	11	0	0	1	1					
	Whiskey Run Spring	84	95	80	4	3	1	1	0	0					
	Lost River Spring	92	86	79	12	11	2	2	0	0					
	Payne Well	81	90	73	7	6	0	0	2	2					
	Vine Well	56	88	49	11	6	2	1	0	0					
		Tetracycline Resistant Genes					Beta-Lactam (including 3rd generation Cephalosporin) Resistant Genes								
ARC associated with resistance phenotypes for <i>E. coli</i>	Sample Site	Total Number of Isolates (n)	<i>tetA</i> (%)	Number of Isolates (n)	<i>tetB</i> (%)	Number of Isolates (n)	Total Number of Isolates (n)	<i>bla</i>CMY-2 (%)	Number of Isolates (n)	<i>bla</i>CTX-M (%)	Number of Isolates (n)	<i>bla</i>DHA (%)	Number of Isolates (n)	<i>bla</i>OXA-1 (%)	Number of Isolates (n)
	ByPass Cave	100	47	47	53	53	97	30	29	69	67	0	0	1	1
	Durbin Well	97	47	46	53	51	106	8	9	88	93	3	3	1	1
	Barren River	108	57	62	43	46	126	19	24	81	102	0	0	0	0
	Carver Well Cave	76	61	46	39	30	84	17	14	83	70	0	0	0	0
	Lost River Rise	94	51	48	59	46	127	22	28	77	98	0	0	1	1
	New Spring	92	52	48	48	44	145	13	19	86	125	0	0	1	2
	Whiskey Run Spring	93	65	60	35	33	64	50	32	47	30	3	2	0	0
	Lost River Spring	86	52	45	48	41	76	43	33	57	43	0	0	0	0

Table 3. *Cont.*

Payne Well	66	55	36	45	30	18	22	4	78	14	0	0	0	0
Vine Well	53	70	37	30	16	52	36	19	60	31	2	1	2	1

Figure 6, complimented by Table 3, depicts the detection frequency of *Enterococcus* spp. isolates resistant to tetracycline antibiotics in relation to landuse spatially, highlighting the extensive prevalence of *tetM* at each site, which is primarily associated with municipal and hospital wastewater, indicating an urban influence to the groundwater system. Over 80% detection frequency of this gene is captured at each site and less than 15% detection frequency of *tetL*, *tetO*, and *tetS* is captured (Table 3). The highest detection frequency for *tetM* is captured at the Payne Well (90%; Table 3) site and the lowest at the Lost River Rise (82%; Table 3) site. The *tetL* gene is captured at each site as well, with the highest frequency at the Lost River Rise (14%; Table 3) site and the lowest at the Whiskey Run Spring (4%; Table 3) site. The *tetO* gene is not detected over 4%, with the Lost River Rise (3%; Table 3) site exhibiting the highest detection frequency (Figure 6). The *tetS* gene also exhibits a low detection frequency with the highest frequency being 3% at the Barren River site (Table 3). Tetracycline is a commonly used antibiotic in both clinical and agricultural practices; however, the high detection frequency of *tetM* throughout the study area indicates a predominant urban influence to the groundwater system.

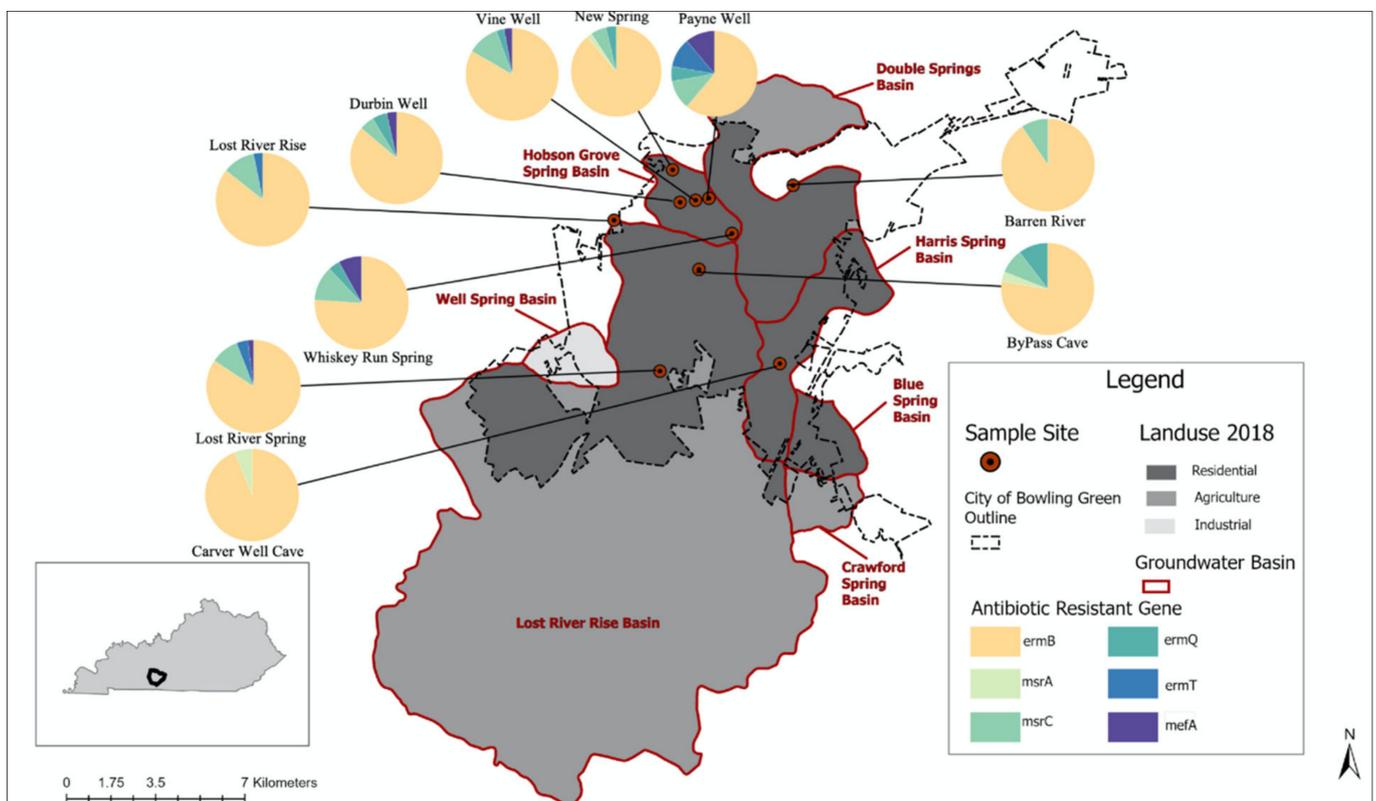


Figure 5. Spatially represented macrolide ARG detection frequency associated with resistance phenotypes for *Enterococcus* spp. and landuse in Bowling Green, KY urban karst groundwater.

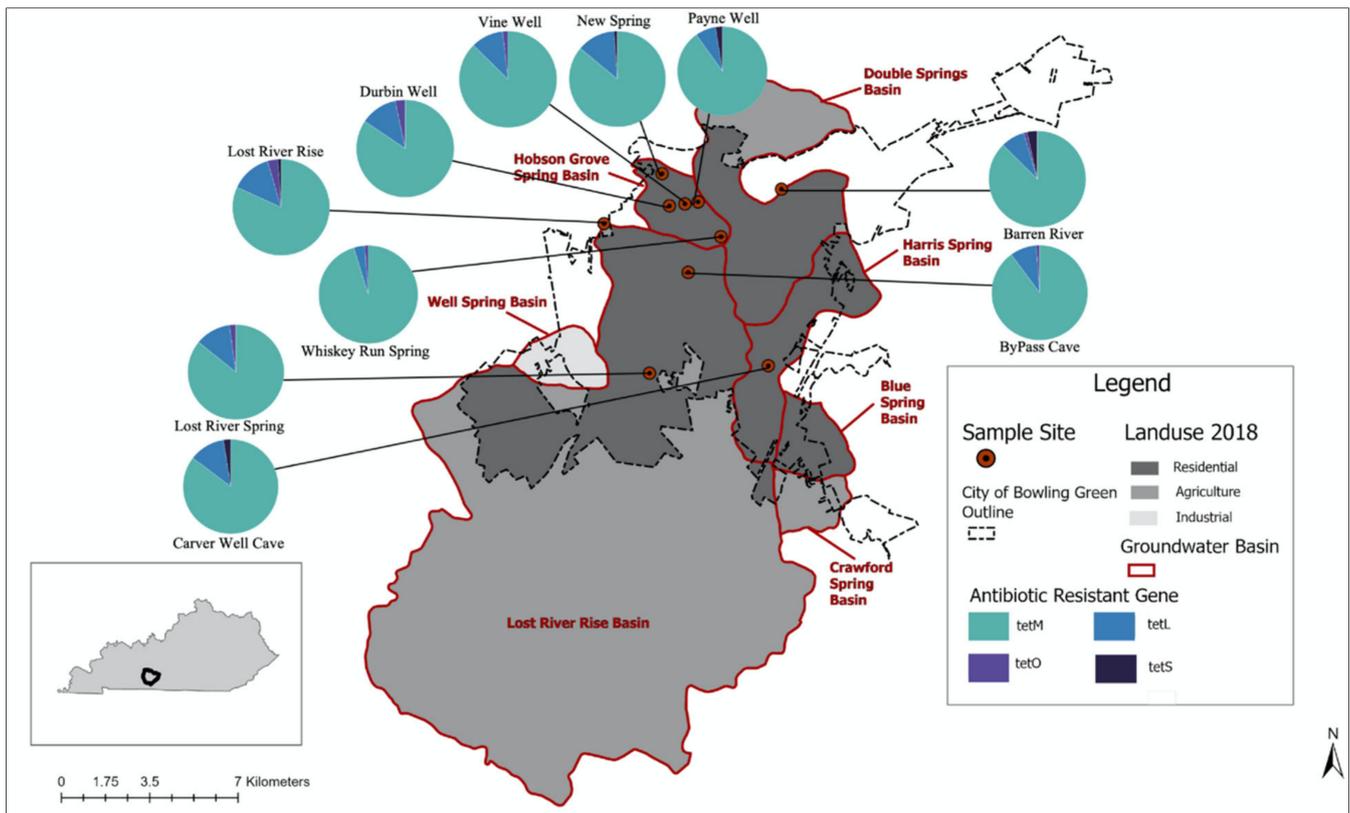


Figure 6. Spatially represented tetracycline ARG detection frequency associated with resistance phenotypes for *Enterococcus* spp. and landuse in Bowling Green, KY urban karst groundwater.

3.2.2. Bowling Green, KY Detection of Antibiotic Resistant Genes Associated with Resistance Phenotypes for *E. coli*

The detection frequency of tetracycline resistant genes in the *E. coli* isolates in relation to landuse is depicted in Figure 7 with detailed percentages in Table 3. The *tetA* and *tetB* genes are both captured at each of the sample sites. The Vine Well site captures the highest detection frequency for *tetA* (70%; Table 3) and the lowest for *tetB* (30%; Table 3). The ByPass Cave and Durbin Well sites capture the lowest detection frequency for *tetA* (47%; Table 3) and the highest detection frequency for *tetB* (53%; Table 3). Similar to the *Enterococcus* spp. Tetracycline-resistant isolates, this antibiotic class is overused and present throughout the environment; however, the association of these two ARGs primarily with untreated sewage, wastewater, and drinking water residuals [26,30,96], indicates a greater urban influence.

The detection frequency of beta-lactam resistant genes, including third generation cephalosporin, within the *E. coli* isolates in relation to landuse, is depicted in Figure 8 and Table 2. The *bla*_{CTX-M} gene is captured at all sample sites with the highest detection frequency at the Durbin Well (88%; Table 3) site and the lowest at the Whiskey Run Spring (47%; Table 3) site. The *bla*_{CMY-2} gene is also captured at all the sample sites with the highest detection frequency at the Whiskey Run Spring (50%; Table 3) site and the lowest at the Durbin Well (8%; Table 3) site. These two genes have been detected throughout the environment and are associated with commonly used antibiotics in this class, highlighting the development of resistance due to extensive overuse. The genes have been detected in agricultural wastewater; however, they are mainly associated with municipal wastewater [26]. The *bla*_{DHA} gene is only detected at the Durbin Well (3%; Table 3), Whiskey Run Spring (3%; Table 3), and Vine Well (2%; Table 3) sites all located in the Hobson Grove Spring Basin predominately influenced by residential landuse (41%; Table 2). The *bla*_{OXA-1} gene is only detected at the ByPass Cave (1%; Table 3), Durbin Well (1%; Table 3), Lost River

Rise (1%; Table 3), New Spring (1%; Table 3), and Vine Well (2%; Table 3) sites. These sites are captured in the Hobson Grove Spring and Lost River Rise basins, which are predominately residential (41%; Table 2) and agricultural (62%; Table 2) landuse, respectively. However, as previously discussed, the ByPass Cave site is directly influenced by commercial and residential landuse, as well as groundwater flowpaths impacted by urbanization (Figure 1). The Lost River Rise site is the outlet for the Lost River Rise basin (62% agriculture; Table 2), receiving a mixture of agricultural and urban impacts throughout the basin; however, the extensive prevalence of the *bla*_{CTX-M} gene indicates greater urban influence.

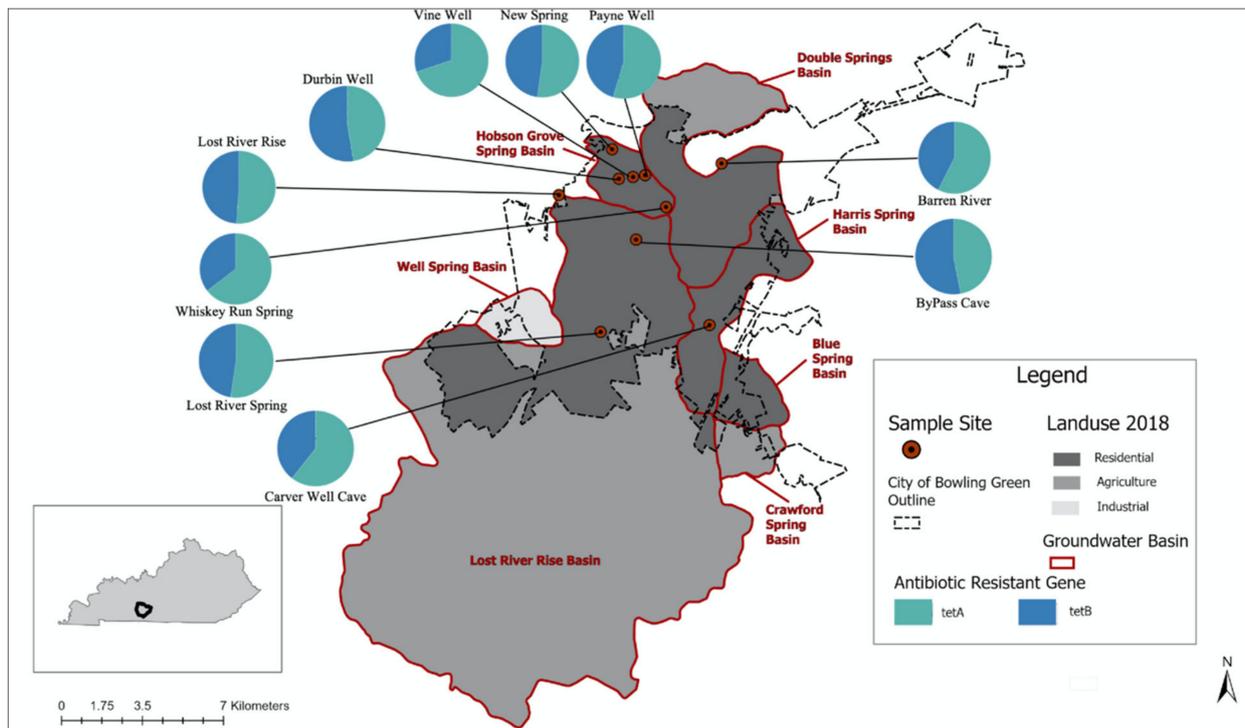


Figure 7. Spatially represented tetracycline ARG detection frequency associated with resistance phenotypes for *E. coli* and landuse in Bowling Green, KY urban karst groundwater.

The study area exhibits mixed landuse within the groundwater basins, but the direct urban influence, including human waste and residual antibiotic compounds from septic systems and leaking sewer systems, is impacting ARG prevalence within the urban karst groundwater system. The detection of agriculturally relevant ARGs (including *msrC*, *ermT*, *ermQ*) at the sample sites highlights the mixed landuse, however, the low detection frequencies of these ARGs indicates the limited influence of agriculture within this system. Agriculturally associated ARGs are detected at sites within the Lost River Rise (Lost River Spring, ByPass Cave, Lost River Rise) and Double Springs (Barren River) basins, which are predominantly agricultural landuse, as well as urbanized basins; however, the urban-associated ARGs present a greater detection frequency. The influence of mixed landuse in karst groundwater was also exhibited in rural karst antibiotic and resistance studies, which detected a lower prevalence of urban-associated antibiotics and resistance [41,49,54,55,57,62,63]. The detection of ARGs throughout the study area indicates an extensive water quality issue, which can negatively impact human health, since these groundwater systems contribute to drinking water resources [41,49].

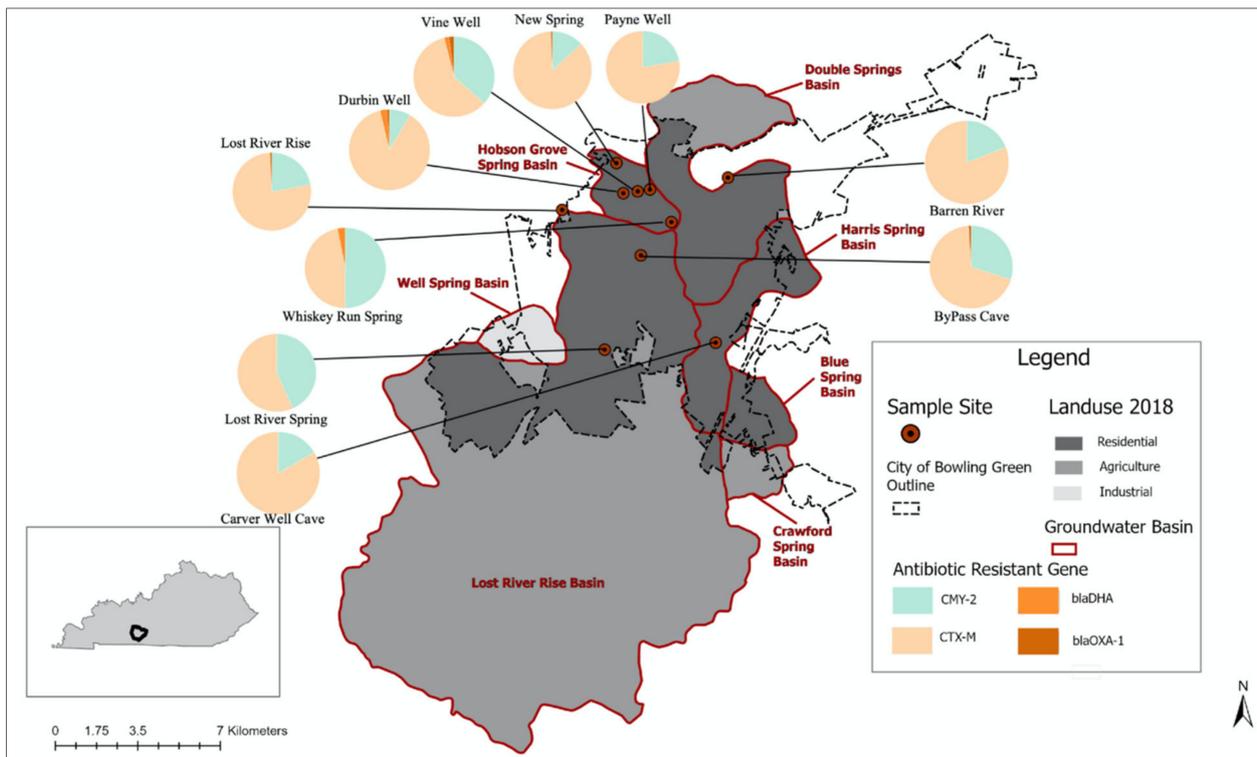


Figure 8. Spatially represented beta-lactam ARG detection frequency associated with resistance phenotypes for *E. coli* and landuse in Bowling Green, KY urban karst groundwater.

3.3. Spatial Overview of Antibiotic Resistant Genes Associated with Resistance Phenotypes and Landuse for Tampa Bay Metropolitan, Florida Urban Karst Groundwater

To further extrapolate the spatial distribution of ARGs associated with resistance phenotypes for *Enterococcus* spp. and *E. coli* detection in urban karst groundwater in relation to land use, the Florida sample sites were analyzed. Similar to Bowling Green study area, this area is predominantly impacted by urban land use (Figure 2); however, the population and land area are greater here. The urban area is mainly residential with impacts to the groundwater from wastewater treatment plants and leaking septic tanks, which can be disseminated throughout the aquifer due to the extensive development of the system and shallowness of the water table. This area has several of the most substantial first-magnitude springs in the state, which are vulnerable and contribute to water quality in the area and beyond. Even though groundwater basins are not delineated in this area, it can be assumed impacts from rural and agricultural land use may be detected at the urbanized karst features due to some of the heterogeneity of the landscape.

The macrolide resistant gene, *ermB*, in the *Enterococcus* spp. isolates is detected at 14% of the sample sites ($n = 6$; Table 4). These sites are influenced by mixed land use, but are located in predominantly urban land use (Figure 9A). The tetracycline-resistant genes, in the *Enterococcus* spp. isolates, are detected at 20 of the 45 sites with 32% detecting *tetM* ($n = 14$; Table 4), 9% detecting *tetL* ($n = 4$; Table 4), 2% detecting *tetO* ($n = 1$; Table 4), and 2% detecting *tetS* ($n = 1$; Table 4). Similar to the macrolide ARGs, the sites with detected prevalence of tetracycline resistance are located in primarily urban areas (Figure 9B).

Table 4. Detection frequency of ARGs associated with resistance phenotypes by prevalence at all sites ($n = 45$) for Tampa Bay Metropolitan Area, FL sample sites.

Antibiotic Resistant Gene Detection Frequency (%)			
	Macrolide Antibiotic	Percent of sites with prevalence (%)	Sites with prevalence (n)
<i>Enterococcus</i> Resistant Genes	<i>ermB</i>	14	6
	Tetracycline Antibiotic		
	<i>tetM</i>	32	14
	<i>tetL</i>	9	4
	<i>tetO</i>	2	1
	<i>tetS</i>	2	1
<i>E.coli</i> Resistant Genes	Tetracycline Antibiotic		
	<i>tetA</i>	14	6
	<i>tetB</i>	18	8
	Beta-Lactam Antibiotic		
	<i>bla</i> CMY-2	11	5
	<i>bla</i> CTX-M	7	3
	<i>bla</i> DHA	0	0
	<i>bla</i> OXA-1	5	2

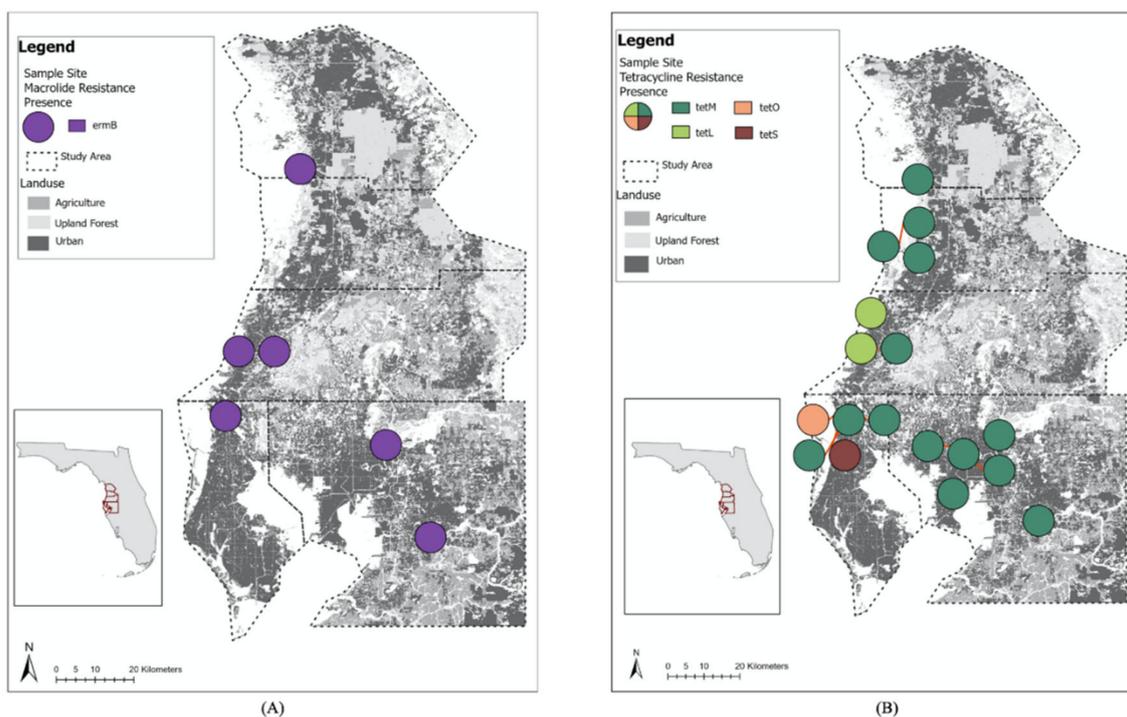


Figure 9. (A) Prevalence of macrolide ARGs associated with resistance phenotypes for all *Enterococcus* spp. in Tampa Bay Metropolitan Area, FL urban karst groundwater. (B) Prevalence of tetracycline ARGs associated with resistance phenotypes for all *Enterococcus* spp. in Tampa Bay Metropolitan Area, FL urban karst groundwater.

The tetracycline-resistant genes, in the *E. coli* isolates, are detected at 14 of the sites with 14% detecting *tetA* ($n = 6$; Table 4) and 18% detecting *tetB* ($n = 8$; Table 4). The beta-lactam-resistant genes, including third generation cephalosporin, in the *E. coli* isolates, are detected at a total of six of the sites, as some sites detected multiple genes (Figure 10B). The *bla*CMY-2 gene is detected at 11% of the sites ($n = 5$; Table 4), the *bla*CTX-M gene is detected at 7% of the sites ($n = 3$; Table 4), the *bla*DHA gene is not detected, and the *bla*OXA-1 gene

is detected at 5% sites ($n = 2$; Table 4). Similar to the other ARGs detected, these sites are developed reflecting an urban influence to the ARB and associated ARGs (Figure 10A,B).

Only a single spot sampling was conducted in the Florida study area; however, the extensive detection of urban-associated ARGs (*ermB*, *tetM*, *tetA*, *tetB*, *bla_{CMY-2}*, and *bla_{CTX-M}*) in both study areas occurred, regardless of sampling resolution, land area, population size and urban impacts, aquifer size, or type of karst features. These findings suggest that regardless of aquifer type, climate, or level of urbanization, these groundwater systems are conducive for ARG development where urban impacts are present. The detection of clinically relevant and wastewater-associated ARGs in developing and well-developed urbanized karst areas indicates urban karst groundwater systems are a potential reservoir and pathway of antibiotic resistance influenced by urban practices. Mixed land use within both study areas does influence the prevalence of ARGs, however, the extensive prevalence of urban-associated ARGs in both areas highlights the strong influence of urbanization. The extensive prevalence detected within this study also highlights the vast dissemination of antibiotic resistance within karst groundwater systems across multiple types and varieties of urban areas. The overall prevalence of bacteria resistant to common antibiotics (tetracycline and macrolide) and last resort antibiotics (beta-lactams, including third generation cephalosporin) within these systems indicates a potential threat to human health from urban karst groundwater systems, which are contributing to the development and dissemination of antibiotic resistance in water resources.

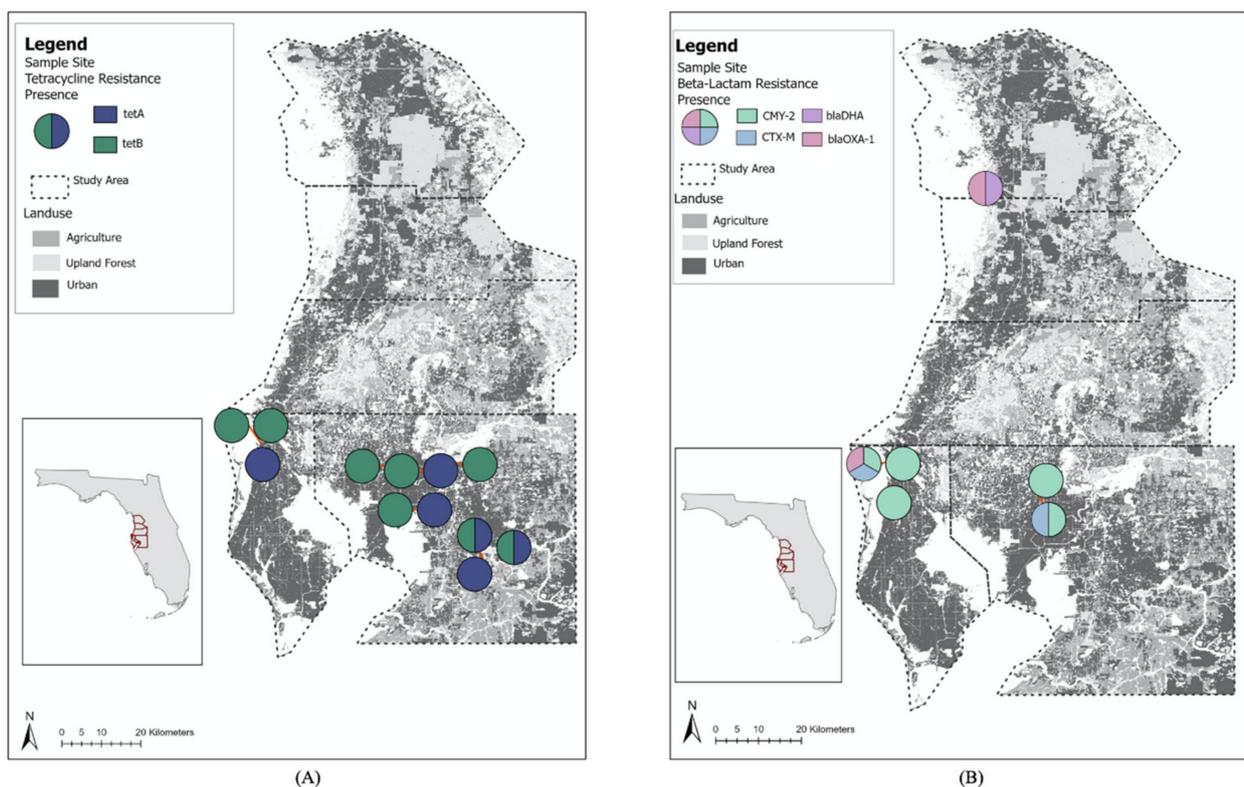


Figure 10. (A) Prevalence of tetracycline ARGs associated with resistance phenotypes for all *E. coli* isolates in Tampa Bay Metropolitan Area, FL urban karst groundwater. (B) Prevalence of beta-lactam, including third generation cephalosporin, ARGs associated with resistance phenotypes for all *E. coli* isolates in Tampa Bay Metropolitan Area, FL urban karst groundwater.

3.4. Temporal Trends of Antibiotic Resistant Genes Associated with Resistance Phenotypes in Bowling Green, KY Urban Karst Groundwater

3.4.1. ARGs Associated with Resistance Phenotypes for *Enterococcus* spp.

Temporal trends of ARGs in karst groundwater have received limited attention; however, the study by Stange and Tiehm captured an increase in unspecific and human-specific MST gene markers in Summer and Fall [63]. ARGs are detected throughout the entire study period for the Kentucky sample sites. A consistent prevalence of macrolide-resistant genes in the *Enterococcus* spp. isolates is present spatiotemporally (Figure 11). Lower detection frequencies during winter are exhibited due to fewer samples collected during this period. The *ermB* macrolide-resistant gene is the most prevalent for this antibiotic class regardless of season and is detected at all sites, with the highest detection frequency for spring season at the Vine Well site (41% of macrolide resistant *Enterococcus* spp. isolates per site by season; Figure 11 and Table S3), for summer season at the Carver Well Cave site (36%; Figure 11 and Table S3), for fall season at the New Spring site (42%; Figure 11 and Table S3), and for winter season at the Payne Well and Barren River sites (11%; Figure 11 and Table S3). The other macrolide-resistant genes (*msrA*, *msrC*, *ermQ*, *ermT*, *mefA*) targeted in this study exhibit low detection frequency, but are still detected throughout the aquifer and the sampling period. Specifically, the detection of *msrC* at the Vine Well site (10%; Figure 11 and Table S3) in the spring season; the Payne Well site (8%; Figure 11 and Table S3) in the fall season; the Lost River Spring (2%; Figure 11 and Table S3), New Spring (2%; Figure 11 and Table S3), and Durbin Well (2%; Figure 11 and Table S3) sites in the winter season; as well as *ermQ* at the Durbin Well site (12%; Figure 11 and Table S3) and *ermT* at the Payne Well site (11%; Figure 11 and Table S3) in the summer season. The consistent and greater detection of macrolide-resistant genes in the *Enterococcus* spp. isolates at primarily residential and commercially influenced sites (Table 2) throughout the sampling period continues to highlight the constant urban influence on ARB and associated ARGs.

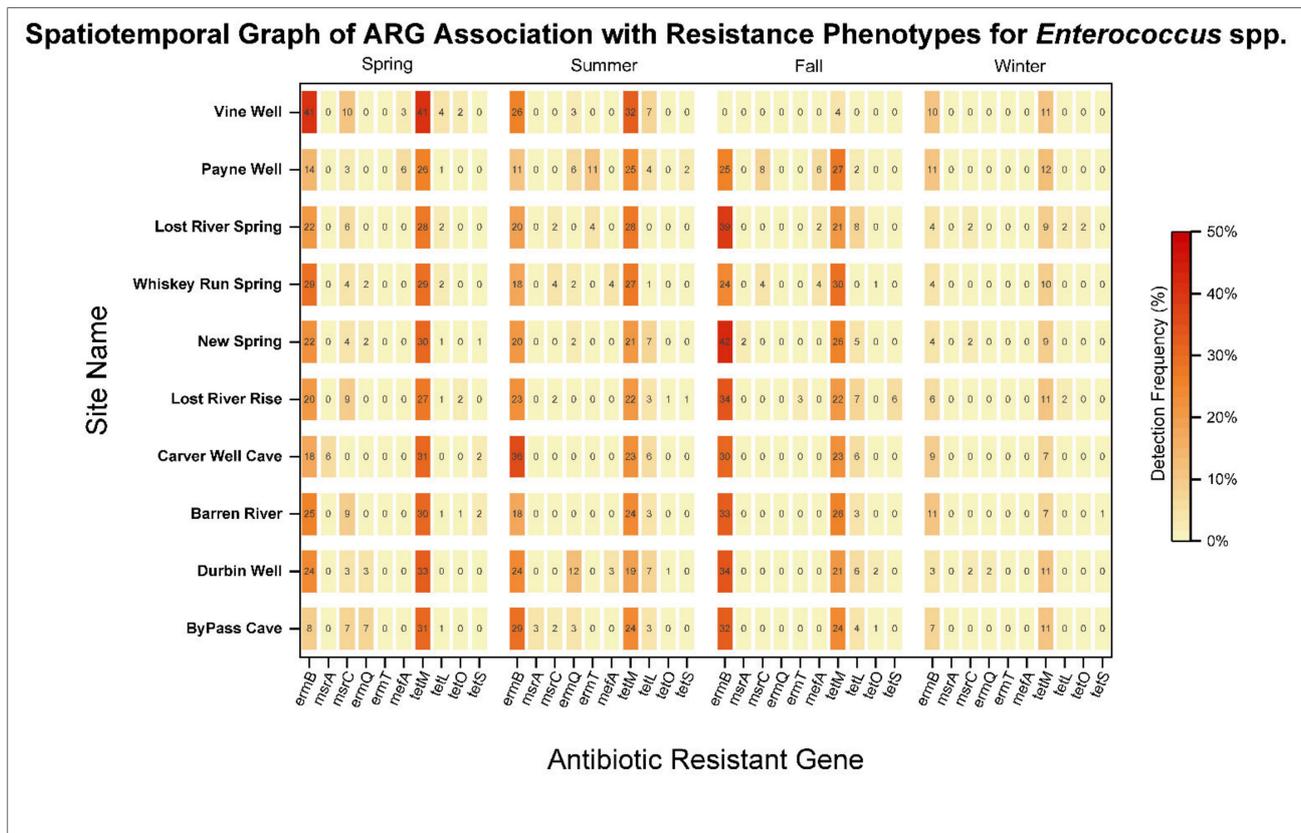


Figure 11. Spatiotemporal trends of ARGs associated with resistance phenotypes for *Enterococcus* spp. isolates.

A constant detection of tetracycline ARGs in the *Enterococcus* spp. isolates is also captured in this study, primarily *tetM*, a municipal and hospital wastewater-associated gene. The Vine Well site has the highest detection frequency for the spring (41%; Figure 11 and Table S3) and summer (32%; Figure 11 and Table S3) seasons. The Whiskey Run Spring site captures the highest detection for fall (30%; Figure 11 and Table S3) and the Payne Well site captures the highest detection for winter (12%; Figure 11 and Table S3). Similar to the macrolide resistant genes in the *Enterococcus* spp. isolates, a greater prevalence is detected at primarily residential impacted sites (Table 2). Seasonal influence at these sites may be related to their shallow nature in the subsurface, as well as their role in discharging heavily impacted water in their basin.

3.4.2. Antibiotic Resistant Genes Associated with Resistance Phenotypes for *E. coli*

A constant prevalence of tetracycline-resistant genes, *tetA* and *tetB*, in the *E. coli* isolates, is detected, primarily at residentially influenced sites, regardless of season (Figure 12). The highest detection frequency for the *tetA* gene is captured at the Vine Well site during spring (32%; Figure 12 and Table S4) and summer (28%; Figure 12 and Table S4). For the fall season, the Barren River and Whiskey Run Spring sites capture the greatest detection frequency for the *tetA* gene (22%; Figure 12 and Table S4), and for the winter season the Lost River Rise site captures the greatest frequency (9%; Figure 12 and Table S4) followed by the Vine Well (8%; Figure 12 and Table S4) and New Spring (7%; Figure 12 and Table S4) sites.

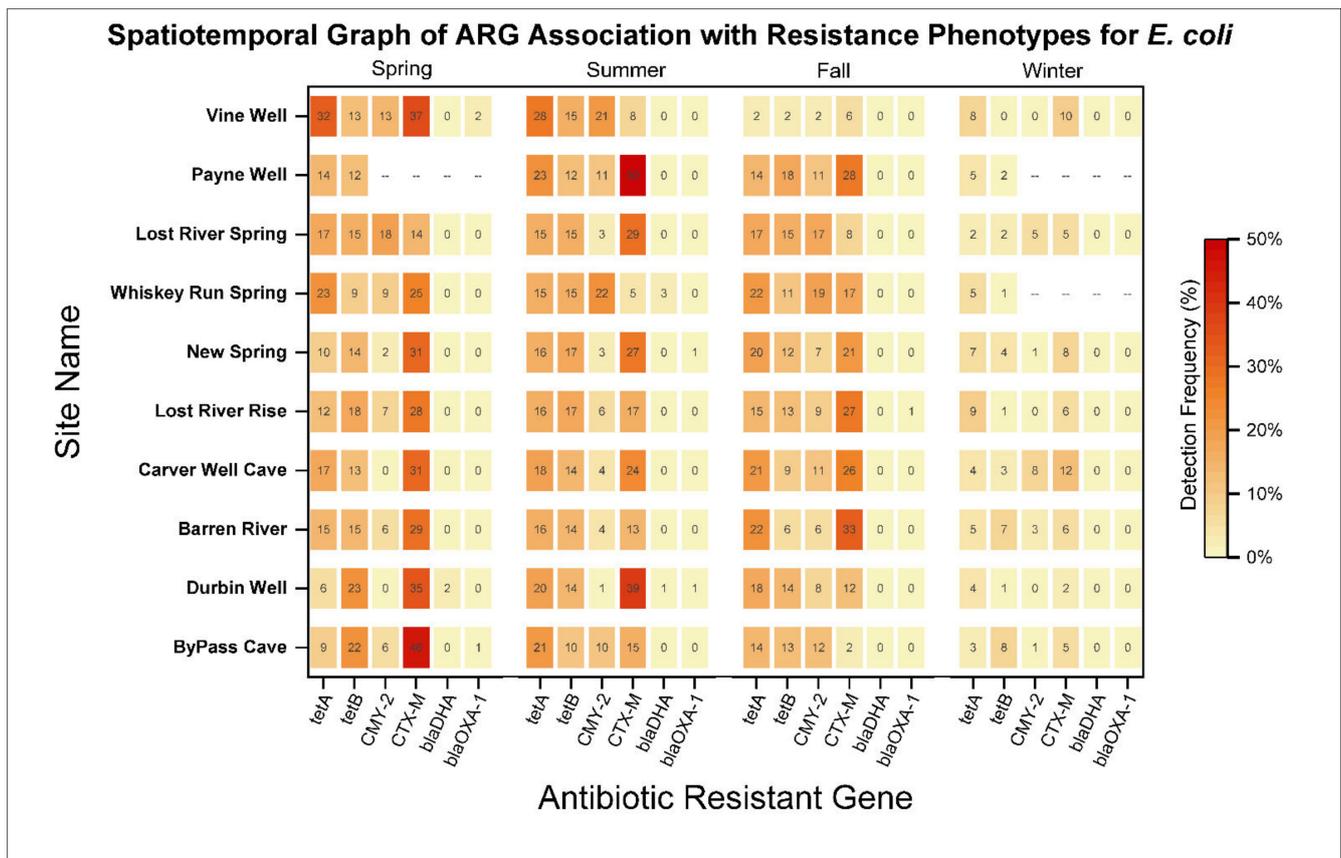


Figure 12. Spatiotemporal trends of ARGs associated with resistance phenotypes for *E. coli* isolates.

The highest detection of the *tetB* gene during spring is captured at Durbin Well (23%; Figure 12 and Table S4) and ByPass Cave (22%; Figure 12 and Table S4). There is a consistent prevalence throughout the aquifer captured for the Summer season (10–17%; Figure 12 and Table S4), and the Payne Well site captures the greatest frequency of *tetB* during Fall (18%; Figure 12 and Table S4). The detection frequencies for the winter season are lower, however, prevalence is still detected, indicated a buffered karst environment aiding in continuous development and dissemination of resistance in the groundwater system.

The beta-lactam, including third generation cephalosporin-resistant genes, in the *E. coli* isolates detected in this urban karst study, are primarily *bla*_{CMY-2} and *bla*_{CTX-M}. The *bla*_{DHA} and *bla*_{OXA-1} genes are detected at four of the sites, but with no more than 3% detection frequency. The prevalence of *bla*_{CMY-2} is detected at each of the 10 sampling sites regardless of season (Figure 12). *bla*_{CTX-M} has the greatest detection frequency for the *E. coli* beta-lactam resistant isolates (Figure 3) and a constant prevalence throughout the sample period and sample sites (Figure 12). For the spring season, the highest detection frequencies are captured at the Vine Well site (37%; Figure 12 and Table S4) and the ByPass Cave site (46%; Figure 12 and Table S4). The summer season demonstrates the greatest frequency (50%; Figure 12 and Table S4) at the Payne Well site, followed by Durbin Well (39%; Figure 12 and Table S4). The highest frequencies during the fall season is 33% at the Barren River site, 28% at Payne Well, and 27% at Lost River Rise (Figure 12 and Table S4). The urban-associated *bla*_{CTX-M} gene has the greatest detection frequency of the targeted ARGs for the winter season (Figure 12), which can indicate an increase in illness and antibiotic use as well as the buffered karst groundwater environment aiding in continuous development and dissemination of ARB and ARGs.

Regardless of seasonal trends, the prevalence of ARGs associated with the resistance phenotypes for *E. coli* and *Enterococcus* spp. are detected throughout the urban karst aquifer. Moreover, unlike surface streams, karst groundwater systems are oftentimes a

buffered environment with respect to sunlight, pH, and temperature, which may create a more hospitable environment for the potential proliferation and dissemination of bacteria year-round [31,47,48,61]. During baseflow conditions, karst systems are also capable of storing pollutants in the epikarst critical zone, including sediments, and urban inputs, such as wastewater, antibiotic compounds, and other ARGs, in high concentrations between storm events, and the extended residence can aid bacteria growth and horizontal gene transfer of ARGs [37,48]. The extensive detection of ARGs in urban karst groundwater systems, regardless of season, highlights the influence of urbanization in these systems, which threatens human health [49,55,63]. Also, the greater detection of urban-associated ARGs (*tetM*, *ermB*, *tetA*, *tetB*, *bla*_{CTX-M}, and *bla*_{CMY-2}) than agricultural ARGs, regardless of mixed land use within groundwater basins, highlights this influence.

3.5. Urban Karst Groundwater and Antibiotic Resistance

Karst groundwater systems are susceptible to contaminants, including bacteria and ARGs, primarily due to the lack of natural filtration from the surface to the subsurface. These pathogens are also directly introduced through urban karst features, such as sinkholes, injection wells, cave entrances, and other surface drainage features [101,102]. Once introduced to the groundwater system, bacteria and ARGs can be rapidly transported throughout the aquifer via the conduit systems, as well as slowly move through the karst matrix having longer residence times [103,104]. In rural karst areas, during extensive baseflow conditions, limited ARGs have been detected, and the increased prevalence of ARGs is associated with precipitation events introducing surface ARGs to the groundwater system, which is then distributed throughout the system and detected at outputs, such as springs [41]. Unlike rural karst systems, the consistent prevalence of ARGs in urban karst groundwater systems is likely due to more widespread and focused contaminant sources, such as leaking septic systems, sewage lines, and WWTPs, which are urbanized in nature [41,105–112]. Also, in karst areas, some homes are not attached to the sewer system and septic tanks or straight injection of waste into caves allows the direct introduction of ARGs to groundwater supplies with no attempt at remediation. This continuous input of bacteria and ARGs aids in the development of the ARB community through vertical and horizontal gene transfer, which can be vastly disseminated during precipitation events [41].

The transport of ARB and associated ARGs within karst groundwater may also be supported through interbasin transfer. Interbasin transfer allows the groundwater to move between basins depending on the aquifer conditions and active flowpaths. Typically, during intense storm events or flooding, the groundwater basin boundaries are altered as the groundwater table rises closer to the surface and activates other flowpaths that allow flow between basins. The vertical flow of contaminants from the epikarst and vadose zones move during storm pulses flushing contaminants down into the phreatic and vadose zones and through the system. The lateral transfer of contaminants within the phreatic and vadose zones occurs within basin boundaries, but during high flow or storm events the phreatic zone boundary may change and the basins may overflow into each other as perched water tables, allowing the overall water table change due to topography and intersecting conduits. Collectively, these hydrologic interactions contribute to the ability of ARB to both develop and move throughout the aquifer due to the interconnectedness of the systems.

Every continent in the world has karst landscape features, with nearly a quarter of the human population living on, or near, karst regions and using karst aquifers as drinking water sources [47,113]. The development of antibiotic resistance is a major concern for public health, particularly in source water, such as karst groundwater, which can directly introduce antibiotic resistance to the community and impact public health [50,51]. This study is only a snapshot of ARGs harbored within cultured bacteria, which does not fully represent the entire resistive population from uncultured bacteria, as well as overlooking extracellular DNA present in the groundwater, which can be disseminated through horizontal gene transfer [114]. This is a major threat to drinking water resources

because the current treatment processes remove bacteria, but not necessarily pathogenic and medically relevant ARGs [50], which can be introduced to the human microbiome through consumption, impacting health, and reintroduced to the karst groundwater system. Further analysis utilizing metagenomic sequencing and high-throughput qPCR are the next steps in understanding the prevalence of antibiotic resistance and the influence of the urbanized karst groundwater system [65]. This is necessary for the development of effective monitoring and regulations to ensure the protection of source water and public health from this human health crisis.

3.6. Limitations and Future Work

The main limitation of this study is the lack of concentration data for the ARB and ARGs detected. Due to the methods utilized and the challenges of culturing water-based bacteria occurring at low concentration, enumeration was not done. The presence or absence data used within this study were able to determine if ARB and associated ARGs are prevalent in urban karst, and a study in progress is expanding on these findings by defining the resistome using high-throughput quantitative PCR (HT-qPCR). Another limitation of this study, which is addressed by the HT-qPCR analysis, is the limited representation of ARB and associated ARGs from culturing techniques. These techniques do not represent all bacteria within the environment and the ARGs captured are specific to cultured bacteria. However, for the purpose of determining prevalence within urban karst systems, this approach was acceptable. When considering the temporal trends of the ARGs, the winter season is not fully sampled as collection did not occur in January. This was taken into consideration when calculating frequencies, and the results were normalized. The Florida study area only has single spot sampling; however, this area is used for confirmation of the ubiquitous nature of ARB and further sampling is needed in the future.

Collectively, there are over 450 urban karst samples indicating the prevalence of ARGs. This study clearly highlights the prevalence of antibiotic resistances within urban karst groundwater systems; however, the influence of environmental parameters, including water quality, has received limited attention thus far. Another study in-progress will address the relationship between water quality and ARGs within these systems to further understand the role of urban karst groundwater as an antibiotic resistance reservoir and dissemination pathway.

A significant limitation in both study areas is the complex nature of karst groundwater. The karst landscape is constantly evolving and the flowpaths respond to the influx of recharge, thereby making it improbable to precisely define the movement of groundwater within a karst system and the sources contributing to the development of antibiotic resistance [72]. The flowpaths and karst feature connections are interpolated, but can change depending on the water table level, recharge rate, and disruption of the landscape from urban development. Also, important to note, the sample sites are not necessarily representative of hydrologic process that could influence the prevalence of antibiotic resistance in other areas of the karst aquifer, such as storage in the epikarst and vadose zones, and associated perched aquifers. This indicates that the sample sites capture only the output of antibiotic resistance within the aquifer, but not the processes in the epikarst and vadose zones within the basin, which may be contributing to development and dissemination [115].

4. Conclusions

The findings from this study highlight urban karst groundwater as an antibiotic resistance reservoir and dissemination pathway. The extensive prevalence of ARGs detected throughout the Bowling Green, KY, a telogenetic karst aquifer, regardless of feature type or groundwater basins, highlights the interconnected nature of the karst aquifer and mixing throughout the system. The detected ARGs are primarily associated with wastewater or clinically relevant antibiotics, indicating a strong urban influence on antibiotic resistance in this system, regardless of the mixed urban and agricultural land use in the groundwater basins associated with this developing urban karst area. The prevalence in this system

is also detected consistently regardless of seasonal variation, indicating ARG storage within the system and the buffered karst groundwater environment contributing to the development and dissemination of ARGs [31,115,116].

To expand the understanding of antibiotic resistance in urban karst groundwater, an eogenetic karst system, the Upper Floridian Aquifer, also highlights urban karst groundwater as a reservoir and dissemination pathway. Regardless of the classification of this aquifer as eogenetic, a larger aquifer and recharge area, and a developed urban karst area, a similar detection of clinically relevant and urban associated genes is detected in both systems, further indicating an urban influence and threat to human and environmental health. The detection in both the Bowling Green and Floridian aquifer also highlights the occurrence of ARGs within these systems, regardless of population size, climate, karst feature, and feature flow capacity.

ARGs are prevalent in urban karst groundwater and potentially threaten human health from exposure as these systems feed surface water springs and rivers that serve as drinking water sources. The extensive dataset within this study aids the understanding of the prevalence of ARB and ARGs in urban karst groundwater systems. This understanding is necessary to influence policies and regulations to ensure the protection and management of this resource for human and environment health.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/w14060960/s1>, Table S1: Bacteria and ARG Primer List, Table S2: Number of resistant isolates for Bowling Green, KY and Tampa Bay Metropolitan Area, FL urban karst groundwater systems defined by antibiotic group and resistance phenotypes, Table S3: Number of *Enterococcus* spp. resistant isolates for Bowling Green, KY urban karst groundwater systems defined by sample site, season, antibiotic group, and resistance phenotypes, Table S4: Number of *E. coli* resistant isolates for Bowling Green, KY urban karst groundwater systems defined by sample site, season, antibiotic group, and resistance phenotypes.

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