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Antibiotic Resistant Gene Concentrations in Bacteria in Groundwater from Agricultural Waste

Koji Barnaby
Western Kentucky University, koji.barnaby212@topper.wku.edu

ANTIBIOTIC RESISTANT GENE CONCENTRATIONS IN BACTERIA IN GROUNDWATER FROM AGRICULTURAL WASTE

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By

Koji Barnaby

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CE/T Committee:

Dr. Jason Polk, Chair

Dr. Rodney King

Dr. Martin Stone

ABSTRACT

The overuse of antibiotics has led to an increase in antibiotic resistant bacteria since some antibiotics are sent into the wastewater system by waste produced by both humans and agriculture, making exposure to these bacteria more likely. The evolution of these bacteria in groundwater is of particular concern, as groundwater is used as a source of drinking water, and infections by these bacteria would be more difficult to treat. There are no policies in place to monitor or regulate antibiotic resistance bacteria in groundwater, leaving the threat to public health unknown. The study area of Crumps Cave in Smiths Grove, Kentucky, located beneath agricultural land, is useful for examining the bacteria in the groundwater to determine if agricultural waste (fertilizer use) has an impact on the antibiotic resistant gene presence in bacteria, particularly *E. coli*. DNA isolated from these bacteria is used to determine the presence of antibiotic resistant genes (ARGs) for common antibiotics, and the exact concentration of certain genes present, using polymerase chain reaction (PCR) and digital droplet PCR (ddPCR), respectively. Trends between the concentration of antibiotic resistant bacteria (ARB) in the groundwater and the disposal of manure can be determined and used to inform best management practices for amendment application in agricultural karst settings.

I dedicate this thesis to my friends and family, who supported me throughout this journey of writing my thesis. I also dedicate this work to people who conduct research to better the world for everyone.

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VITA

EDUCATION

Western Kentucky University, Bowling Green, KY — December 2021
B.S. in Biochemistry – Mahurin Honors College Graduate
Honors CE/T: *Antibiotic Resistant Gene Concentrations in Bacteria in Groundwater from Agricultural Waste*

Bowling Green High School, Bowling Green, KY — May 2017

PROFESSIONAL EXPERIENCE

HydroAnalytical Lab, WKU — Sept. 2019-May 2021
Student Analyst

Department of Chemistry, WKU — Aug. 2019-May 2021
Laboratory Teaching Assistant

AWARDS & HONORS

Summa Cum Laude, WKU, December 2021
Honors Development Grant, WKU, October 2020
Outstanding Musician Award, Harlaxton College, December 2018

PROFESSIONAL MEMBERSHIPS

American Chemical Society (ACS)

INTERNATIONAL EXPERIENCE

Harlaxton College, Lincolnshire, United Kingdom — Aug. 2018-Dec. 2018
Semester Long Study Abroad

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INTRODUCTION

Access to clean water is a universal right. Global organizations, such as the United Nations (UN), recognize this and have designated the "availability and sustainable management of water and sanitation for all" a Sustainable Development Goal (SDG) for 2030 (United Nations Statistics Division, 2021, p.38). However, at the moment, the world is not likely to meet the targets set by this SDG due to water pollution and climate change (United Nations Economic and Social Council, 2021). The combination of agricultural, consumer, industrial, and sewage wastes pollute the water and affect its quality by adding unnatural and possibly harmful chemicals to the system. These contaminants create problems for those who use that water, such as illnesses in humans and uninhabitable conditions for animals and plants.

Issues with water quality affect the human population. Certain situations directly cause health problems, such as chemical poisoning or bacterial infections from consuming contaminated water. The Flint Water Crisis in Flint, Michigan is a prime example of a water quality dealing with chemical poisoning. The water was contaminated with lead from the pipes, so the people who consumed this water were exposed to lead. Lead poisoning has several effects on the body, particularly the nervous system, where it causes learning and developmental disabilities and memory problems (Zahran et al., 2017). Thus, those affected by this water quality issue will have long-term consequences due to the contaminated water.

A well-known example of a water quality issue due to bacteria is the cholera outbreak of 1854 in London, England. The River Thames, the water source of the area, was contaminated with human waste when the government had the overflowing cesspools dumped into it. More than 600 people died due to the cholera outbreak. A physician determined that the outbreak was due to a certain water pump that contained the bacteria, which led to cholera infections, and the pump was removed to prevent more spreading of cholera (Paneth et al., 1998). The effects of this outbreak led to improvements in sanitation.

The rise in the use of personal care products and pharmaceuticals (PCPP) by individuals, in healthcare, and in agriculture has contributed to the rapid growth of antibiotic resistant bacteria (ARB) in environmental systems. Global organizations, such as the World Health Organization (WHO) and UN, have recognized these ARB as emerging pathogens because infections associated with ARB will become more difficult to treat with current methods (WHO, 2015, p.1-3; United Nations Environment Programme, 2017, p.12-13). The Centers for Disease Control and Prevention (CDC) reported that approximately 3 million people in the United States face an antibiotic resistant infection each year (2019, p.6) Because wastewater treatment plants are not able to effectively remove these PCPPs, these compounds remain in the water systems and can lead the rise of ARB in drinking water sources such as groundwater (Gogoi et al., 2018). Consuming water containing ARB directly exposes the body to the bacteria that can lead to more severe infections. Thus, understanding the prevalence of ARB in drinking water sources like groundwater is necessary for preventing these severe antibiotic resistant infections.

Karst landscapes store this groundwater in aquifers that were developed by the dissolution of soluble rock such as limestone (Ford and Williams, 2007). These areas are

ubiquitous, existing under approximately 20% of the Earth's surface and on nearly every continent (Reed et al., 2010). Importantly, these areas store over 25% of the world's population's drinking water (Anaya et al., 2014); approximately 40% of the drinking water in the United States comes from these aquifers (Ford and Williams, 2007). Therefore, contamination of these aquifers with human, industrial, and agricultural waste products would lead to major water quality and public health crises.

The purpose of this study was to identify the presence and to quantify the absolute concentrations of various antibiotic resistant genes (ARGs) in the extracted DNA by utilizing endpoint PCR and ddPCR, respectively. The DNA was isolated from bacteria collected from a karst groundwater system. This karst area is surrounded by agricultural land use and not by urban areas, so the potential effects of agriculture on ARG concentration can be determined without the effects of an urban environment. The following research questions of this study include:

1. Are ARGs present in this karst groundwater system, and if so, what are their concentrations?
2. Assuming ARG concentrations vary, what are the environmental and agricultural influences that may affect them?

LITERATURE REVIEW

Agriculture and Karst

Groundwater is water that exists below the surface of the Earth in pores of soil and rock. It is stored in underground aquifers that are largely untouched by outside forces, so this water is readily used as a source of drinking water (Anaya et al., 2014). However, because waste generated from industrialization and urbanization can enter groundwater systems easily, there is a growing concern of contamination of this water source that would alter the quality of water. One of the main sources of groundwater pollution comes from agricultural waste, and a growing public health concern is regarding antibiotic resistance bacteria in karst groundwater sources.

A major challenge in karst aquifers is they are vulnerable and prone to contamination. The water is not filtered by the bedrock to remove these contaminants due to its porous and highly connected nature between the surface and subsurface (Vesper et al., 2003). Moreover, because the surrounding land of sinkhole plains is typically used for agriculture and septic systems are used for disposing human waste, the influx of fertilizers, livestock waste, and septic waste into karst aquifers can occur easily (Panno et al., 2001). The contaminated groundwater in karst aquifers can flow into springs, wells, and surface waters; therefore, the water used in these areas for consumption can lead to health issues, such as bacterial infections, with increased resistance to current antibiotics.

The agricultural practice of applying animal waste as amendments for nutrient enhancement can lead to groundwater pollution if they are not fully utilized by crops. These pollutants introduce bacteria and nutrients necessary for bacterial growth into the

groundwater system. Nitrates, associated with agricultural land use, are involved in processes like nitrification and denitrification in bacteria. The application of inorganic and organic fertilizers can enter the soil and eventually can contaminate the groundwater. Because these karst areas are prone to contamination due to a lack of filtration, nitrate levels have been reported to be higher in groundwater basins that have agricultural land use on the surface (Neill et al., 2003). Additionally, carbon dioxide (CO_2) and methane (CH_4) are used in microbial metabolism. These gases enter the cave through the soil where they are produced due to the decaying of organic matter (Bakalowicz, 2004). The introduction of these nutrients from agricultural waste into the groundwater creates an environment where bacteria can proliferate.

Best management practices (BMPs) are farming methods that lower the risk of contamination while improving crop yield. Due to the uniqueness of karst regions, these BMPs should be tailored to the specific needs and relevance of each site (Bakalowicz, 2011). In 1994, the Kentucky General Assembly passed the Kentucky Agriculture Water Quality Act (KRS. 224.71-100 through 224.71-140) in order to protect groundwater from pollution from agricultural activities. But the complexity of the karst hydrology and its role in carrying contaminants through the system is difficult to address (Vanderhoff, 2011). Additionally, antibiotics and other pharmaceuticals are not regulated, making it difficult to control the ARB populations that exist in groundwater systems (Doummar et al., 2018).

Emerging Pathogens

Consuming contaminated water is a public health and water quality issue due to the direct exposure of pathogens from water. These pathogens can stem from introducing personal care products and pharmaceuticals (PCPP) to the environment. These products

become present in the environment through direct sources such as patient excretions and livestock activities and indirect sources like untreated wastewater. PCPPs in the environment are not regulated, allowing these pollutants to remain in the system (Doummar et al., 2018). It has been shown that antibiotic pollution in the environment facilitates the rise of ARB; therefore, the overuse of antibiotics will lead to more resistant ARB. However, it is difficult to remove these products because these molecules can be in different forms after being processed by the body. To have total elimination, processes would have to be coupled, which would be too costly to implement. Yet there is currently no method to remove these contaminants (Courtier et al., 2018), so they remain in the environment where they can lead to more ARB.

There is an overuse of pharmaceuticals in some agricultural practices for livestock (non-organic practices). Medicines like antibiotics and hormones are mainly used for the promotion of growth in animals. Thus, the waste produced by these animals contains antibiotic residues and ARB (Subbiah et al., 2011). Using this waste as manure for fertilization, ARB from the manure can be introduced to the soil bacteria, leading to ARB growth. Additionally, it has been shown that when manure produced by animals not treated with antibiotics was used as fertilizer, there was an increase in ARB in the soil, suggesting that ARB were already present from previous manure fertilization and proliferated due to the nutrient enrichment from the manure (Agga et al., 2015). So, ARB are present in the soil that has been fertilized with manure from animals treated with antibiotics for their growth and these bacteria can enter groundwater systems through agricultural runoff and infiltration (Pruden et al., 2013).

Agricultural amendments are when new natural fertilizer is added to the existing soil for nutrient enrichment. These fertilizers are typically derived from animal manure. While these amendments are useful for planting crops, they can also facilitate ARB proliferation in the soil by providing essential nutrients (Agga et al., 2015). Continuous application of these organic amendments to the soil has led to increased survivability of some bacteria in groundwater conditions (Filip et al., 1988). Therefore, these amendments can be directly introduced into the aquifers during storm events, and because there is no filtration system for these karst aquifers, these nutrient and bacterial contaminants may lead to an increase in ARB in the water (Vanderhoff, 2011).

Antibiotic Resistance in Bacteria

The rise in ARB can be attributed to the overuse of antibiotics in both healthcare and agricultural setting (Vikesland et al., 2018). While bacteria can develop antibiotic resistance through random mutations that can occur during DNA replication, there are distinct methods through which bacteria can become antibiotic resistant facilitated by the environment. When bacteria acquire a mutation that causes them to be resistant to a given antibiotic, that mutation and the bacteria associated with this favorable mutation are selected by the antibiotic because those without the mutation die, and the resistant bacteria continue to grow and proliferate. The resistance that stemmed from mutations and selection is passed onto subsequent generations through vertical gene transfer. Bacteria can also develop resistance by acquiring genes from other, already resistant bacteria or via a process called horizontal gene transfer. This process can occur between different bacteria species, unlike vertical gene transfer, and utilizes conjugation, transduction, and transformation to exchange the genes. Notably, ARB present in the environment may transfer the ARGs to

pathogens via horizontal gene transfer to create pathogens resistant to current treatments (Tenover, 2006; Vikesland et al., 2018).

Bacteria that have acquired this resistance have shown different methods of limiting antibiotic efficacy. One method is the enzymatic degradation of the antibiotic and can occur when the bacteria have the genes that encode for these enzymes like β–lactamases. Bacteria may also have genes that encode for the production efflux pumps to remove the antibiotic from the cell before reaching the target site. The bacteria may gain genes that change their cell wall to decrease permeability or to no longer contain the antibiotic binding site (Tenover, 2006; Vikesland et al., 2018); therefore, a mutation that causes resistance or acquiring a gene that causes resistance can lead to rapid bacterial adaptation and increased survivability from the introduction of antibiotics to their environment.

ARB in drinking water sources, such as groundwater, is a serious problem because there is little control in how these bacteria spread. To prevent a widespread outbreak of an illness stemming from groundwater pollution, knowing how to avoid this type of pollution and its effects are necessary (Hao et al., 2018). It is difficult to remove antibiotic resistant bacteria from groundwater in karst environments because there is no buffer of organic matter that removes these bacteria, unlike in other porous media aquifers (Vesper et al., 2003). Moreover, with the introduction of nutrients from the fertilizer or soil, these ARB can proliferate, and bacteria that are not antibiotic resistant can gain this resistance through the aforementioned methods in the groundwater (Agga et al., 2015). Thus, the antibiotic resistant bacteria stay in the system and can eventually end up in drinking water sources (Andrade et al., 2020). Currently, no studies have examined the potential connection between agricultural activities and ARB in karst groundwater systems.

STUDY AREA

Samples were taken from Crumps Cave located in the Smiths Grove area of Warren County, Kentucky. This cave was selected because the land surrounding Crumps Cave is used in agricultural practices to examine if the agricultural waste has an impact on the antibiotic resistant gene expression by the bacteria in the groundwater. Figure 1 shows an overlay of the cave with the surrounding agricultural land on the surface, which is primarily row crops, such as soybeans and corn. Crumps Cave is part of a karst area known as the Pennyroyal Plateau sinkhole plain. The Pennyroyal Plateau sinkhole plain makes up the Western Pennyroyal Karst region along with the Mammoth Cave Plateau in southcentral Kentucky. Also, this cave is found in the upper part of the St. Louis Limestone (Tobin et al., 2020).

Figure 1. Crumps Cave Overlay with Surrounding Area (Source: Vanderhoff, 2011).

The karst environment and in-cave waterfall make it an ideal source of groundwater. This waterfall, named waterfall 1 (WF1), is approximately 40 m from the entrance in Crumps Cave. Figure 2 shows a plan view of the cave with WF1 marked with the black circle. WF1 is roughly 4.5 m in height and is 25 m below the surface where agricultural practice takes place. The waterfall drains water from the bottom of the epikarst on the ceiling of the cave. There are also a number of monitoring stations located on the surface that produce high-resolution data such as a weather station, three soil lysimeters at depths of 10 cm, 20 cm, and 30 cm, and two wells: a shallow one approximately 15 m deep and a deep one approximately 50 m deep. The shallow well penetrates the epikarst while the deep well penetrates regional aquifers. This water eventually flows into the Barren River, which is the water supply for the surrounding area and the City of Bowling Green.

Crumps Cave is located in Warren County, Kentucky in a humid subtropical climate. The temperature lies between 14.1 ºC in the fall and 15.0 ºC in the spring with the average annual temperature being approximately 14.4 ºC. Precipitation rates here average approximately 276 cm per year (NOAA, 2021).

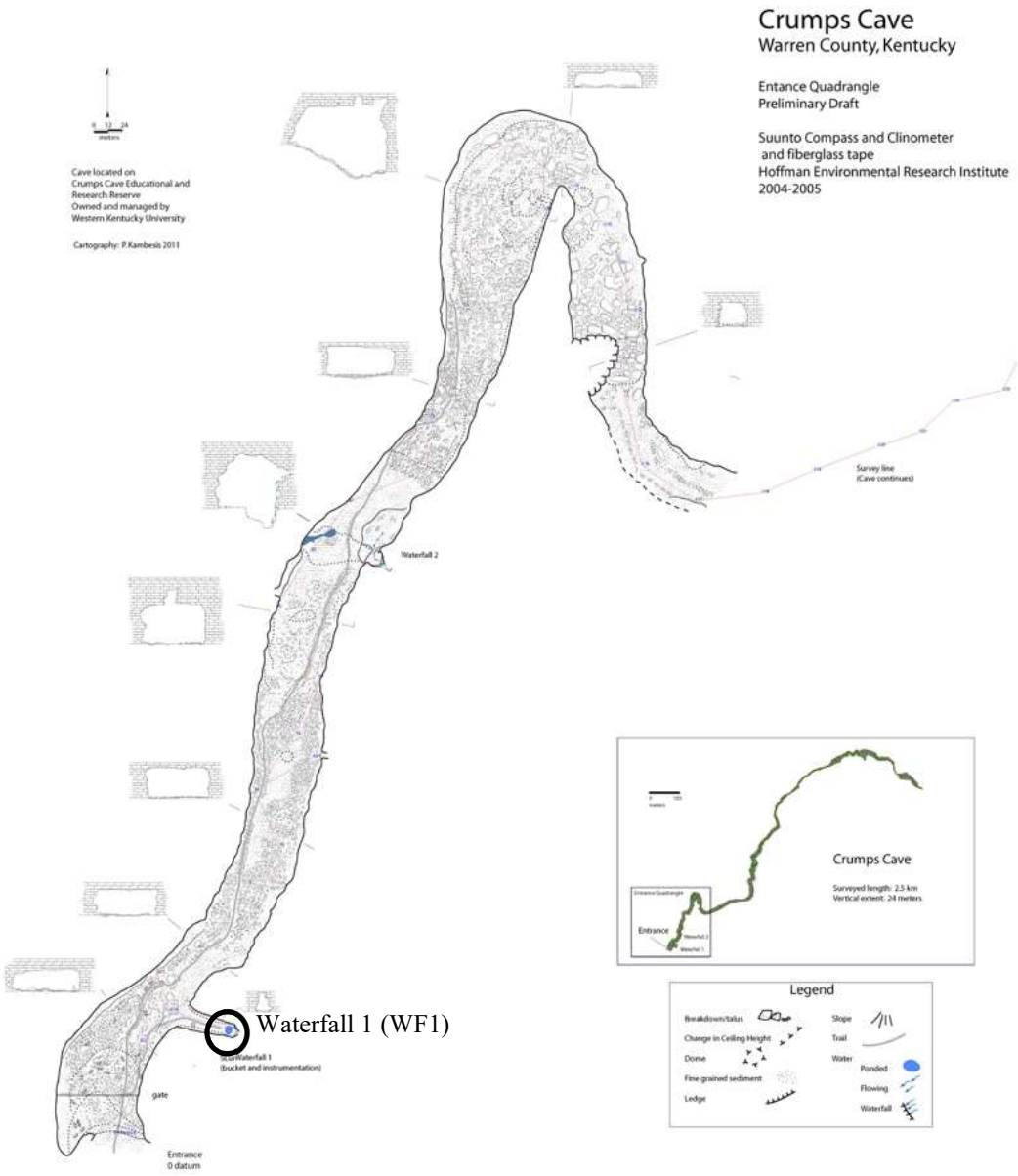

Figure 2. Location of Study Area at Crumps Cave.
(Source: Modified from Polk et al., 2013)

METHODOLOGY

Data Collection

Samples were collected weekly from September 30, 2015, until September 26, 2017, from WF1 in Crumps Cave. Additional high-resolution sampling events took place on May 16, 2016, and May 5, 2017, where samples were collected every hour over a six-hour period. A 500 mL plastic bottle, which was acid-washed and sterilized by autoclaving prior to sampling, was directly filled with the sample for bacterial isolation and DNA extraction. A range of 20 mL – 500 mL from the collected sample, determined by its turbidity, was filtered onto 0.2 mm white polycarbonate filters (Millipore, Billerica, MA, USA). The filters were placed into a lysing matrix tube from the FastDNA Spin kit for soils (MP Biomedical, Solon, OH, USA). The bacterial DNA was extracted according to the manufacturer's specifications and stored at -20 ºC.

High-resolution geochemical data were gathered in addition to sampling at WF1. A HOBO U-30 weather station on the cave surface collected data in 10-minute intervals for temperature, relative humidity, barometric pressure, rainfall (mm/10 mins), and wind speed. A YSI 556 multiparameter handheld instrument and later a YSI ProDSS (digital sampling system) multi-parameter handheld were used to measure pH (±0.2 units), specific conductivity (SpC) (±0.001 mS/cm), temperature (±0.15 ºC and ±0.2 ºC, respectively), and dissolved oxygen (DO) (±0.1 mg/L). The YSI reservoir was filled with 100 mL for these data readings. An EXO II data logger sonde accumulated high-resolution, hydrogeochemical parameters in 10-minute intervals (temperature, pH, specific

conductivity, turbidity). Lastly, a HOBO transducer collected the temperature and pressure of a discharge bucket under the waterfall to determine discharge rate (L/s).

Sample Analysis

Endpoint polymerase chain reaction (PCR) was used to determine the presence of certain ARGs in the extracted DNA. The genes, primers, and annealing temperatures for each assay are shown in Table 1. The PCR was done using a mixture that contained 10.0µL of 2x HotStarTaq *Plus* Master Mix (Qiagen, Hilden, Germany), 1.0 µL of 10 µM (10.0 pmol) of the sense (F in Table 1) primer, 1.0 µL of 10 µM (10.0 pmol) of the anti-sense (R in Table 1) primer, 9.5 µL of RNA-ase Free Water (dH_2O), 2.5 µL of 10x CoralLoad Concentrate loading dye (Qiagen) for visualizing bands during gel electrophoresis, and 1.0 µL of the undiluted DNA sample. $bla_{CTX\text{-}M}$ and $bla_{CMY\text{-}2}$ were analyzed with duplex PCR. The volume of water in the reaction mixture was adjusted to account for the two sets of primers.

The following program was carried out on a PTC-200 (MJ Research, Poway, CA, USA) and an Applied Biosystems 2720 Thermal Cycler (Thermo Fisher Scientific Inc., Waltham, MA, USA) for these PCR assays: 95 ºC for 5 mins, 30 cycles of 95 ºC for 30 s, the annealing temperature for the specific gene shown in Table 1 for 30 s, and 72 ºC for 30 s, then 72 ºC for 10 mins, and refrigerated at 4 ºC. After the PCR program finished, the results of the reaction were viewed through gel electrophoresis. Then, 15 µL of the PCR product was pipetted into the wells of the gel, and 10 µL of the Fisher BioReagents exACTGene 100bp PCR DNA Ladder was loaded into the gel (Waltham, MA, USA). For the duplex reaction, 10 µL of the exACTGene 1kb Plus DNA Ladder from Fisher Scientific was loaded into the gel (Waltham, MA, USA). The gels were run for 2 hours with a Fisher

Biotech Electrophoresis System and a Thermo Scientific EC 3000 XL Power Supply (Waltham, MA, USA) and visualized on a UVP BioSpectrum MultiSpectral Imaging System (Analytik Jena, Upland, CA, USA).

Table 1. Primers for Target ARGs and Sequences in Endpoint PCR.

Antibiotic Class	Target Gene	Primer	Sequence (5' - 3')	Annealing Temp (°C)	Product (bp)	Reference
Cephalosporin	*bla* $_{CTX-M}$	CTX-M-F CTX-M-R	CCGCTGCCGGTYTTATC ATGTGCAGYACCAGTAA	55	512	Cottell et al., 2013
Cephalosporin Cephamycin	*bla* $_{CMY-2}$	CMY-2-F CMY-2-R	GACAGCCTCTTTCTCCACA TGGAACGAAGGCTACGTA	55	1015	Gray et al., 2004
Fluoroquinolone	*qnrA*	qnrA-Fu qnrA-Ru	TTCTCACGCCAGGATTTG CCATCCAGATCGGCAAA	60	521	Guillard et al., 2011
Fluoroquinolone	*qnrB*	qnrB-Fu qnrB-Ru	GGMATHGAAATTCGCCACTG TTYGCBGYYCGCCAGTCG	60	261	Guillard et al., 2011
Fluoroquinolone	*qnrC*	qnrC-Fu qnrC-Ru	ATTACGGGTTGTAATTTGTCTTATG ATCAGAAAATGATCCCCTACT	60	144	Guillard et al., 2011
Fluoroquinolone	*qnrD*	qnrD-Fu qnrD-Ru	GGAGCTGATTTTCGAGGG AGAAAAATTAGCGTAACTAAGATTTGTC	60	105	Guillard et al., 2011
Fluoroquinolone	*qnrS*	qnrS-Fu qnrS-Ru	GTGAGTAATCGTATGTACTTTTGC AAACACCTCGACTTAAGTCT	60	169	Guillard et al., 2011
Fluoroquinolone	*qepA*	qepA-Fu qepA-Ru	GCCGGTGATGCTGCTGA CAGRAACAGCGCSCCSA	60	214	Guillard et al., 2011
Aminoglycoside	*aadA1*	aadA1-F aadA1-R	GCGAGCTTTGATCAACGACC ATGTCATTGCGCTGCCATTC	60	150	Vikram et al., 2017

Quantitative PCR (qPCR) was done to determine the specific concentration of the targeted gene. These assays were done utilizing droplet digital PCR (ddPCR). ddPCR provides absolute quantification of the target product and is highly sensitive due to the independent reactions occurring through in each of the 20,000 droplets in each sample well. The genes, primers, and annealing temperatures for these assays are shown in Table 2. The reactions were mixtures that contained 10.0 µL of 2x QX200 ddPCR EvaGreen Supermix from Bio-Rad (Hercules, CA, USA), 1.0 µL of 10 µM of the sense primer, 1.0 µL of 10 µM of the antisense primer, 12.0 µL of dH_2O, and 1.0 µL of the undiluted DNA sample. The plate was sealed with the PX1 PCR Plate Sealer and was thoroughly vortexed and centrifuged for 30 s to ensure proper mixing and to remove any bubbles that formed.

Utilizing the QX200 Automated Droplet Generator, the estimated 20000 reaction droplets were formed in a new plate by taking 20 µL from the reaction plate and 20 µL of the EvaGreen Oil. This plate was sealed, and the following PCR program was run on a C1000 Touch Thermal Cycler: 95 ºC for 15 mins, 40 cycles of 95 ºC for 30 s and the annealing temperature for the specific gene shown in Table 2 for 1 min, then 4 ºC for 5 mins, 90 ºC for 5 mins, and refrigerate at 4 ºC. The plates were then read using the QX200 Droplet Reader. The results were analyzed using the QuantaSoft Software, and threshold values were set to discern the target gene concentrations.

Table 2. Primers for Target ARGs and Sequences in ddPCR.

Antibiotic Class	Target Gene	Primer	Sequence (5'-3')	Annealing Temp (°C)	Product	Reference
Tetracycline	*tet(A)*	tet(A)-F tet(A)-R	CGGCAATCATTCCGAGCATG ATTCTGCATTCACTCGCCCA	60	90bp	Vikram et al., 2017
Tetracycline	*tet(B)*	tet(B)-F tet(B)-R	TGGTGGTGGGATCGCTTTAC AATGGGCCAATAACACCGGT	60	125bp	Vikram et al., 2017
Tetracycline	*tet(M)*	tet(M)-F tet(M)-R	GTGCCGCCAAATCCTTTCTG GCATCCGAAAATCTGCTGGG	60	249bp	Vikram et al., 2017
Erythromycin	*erm(B)*	erm(B)-F erm(B)-R	TCACCGAACACTAGGGTTGC CTGTGGTATGGCGGGTAAGT	60	130bp	Vikram et al., 2017
Sulfonamide	*sul1*	sul1-F sul1-R	CGCACCGGAAACATCGCTGCAC TGAAGTTCCGCCGCAAGGCTCG	56	163bp	Pei et al., 2006
Sulfonamide	*sul2*	sul2-F sul2-R	TCCGGTGGAGGCCGGTATCTGG CGGGAATGCCATCTGCCTTGAG	61	190bp	Pei et al., 2006

The total concentrations of *E. coli* and *Enterococci* were also identified with ddPCR. The primers, annealing temperatures, and probes for these assays are shown in Table 3. The mixture for these Probe reactions contained 10.0 µL of ddPCR Supermix for Probes (No dUTP) from Bio-Rad, 1.5 µL of 10 µM of the sense primer, 1.5 µL of 10 µM of the antisense primer, 1.0 µL of 5 µM of the specific probe, 10.0 µL of dH_2O, and 1.0 µL of the undiluted DNA sample. The procedure was the same as the EvaGreen assays except for the utilization of the Probe Oil in place of the EvaGreen Oil in the Droplet Generator.

The PCR program used for these reactions was: 95 ºC for 10 mins, 40 cycles of 95 ºC for 30 s and 60 ºC for 1 min, then 98 ºC for 10 mins, and refrigerate at 12 ºC.

Table 3. Primers for Target Genes and Sequences in Probe ddPCR.

Target Gene	*uidA* (*E. coli* Beta-Glucuronidase)	
Annealing Temp (°C)	60	**Sequence (5'-3')**
Forward Primer	784F	GTGTGATATCTACCCGCTTCGC
Reverse Primer	866R	AGAACGCTTTGTGGTTAATCAGGA
Probe	EC807 FAM-TAMARA	TCGGCATCCGGTCAGTGGCAGT
Product (bp)	82bp	
Reference	Frahm et al., 2003	
Target Gene	ENT 23S rRNA gene	
Annealing Temp (°C)	60	**Sequence (5'-3')**
Forward Primer	ECST748-F	AGAAATTCCAAACGAACTTG
Reverse Primer	ECST854-R	CAGTGCTCTACCTCCATCATT
Probe	Enterococci-Gl813tQ	TGGTTCTCTCCGAAATAGCTTTAGGGCTA
Product (bp)	106bp	
Reference	Frahm et al., 2003	

Source-specific *Bacteroides sp.* were also determined using ddPCR. These bacteria are specific to the host's digestive systems, so targeting genes associated with them indicates if there was contamination from fecal waste. The reactions contained a mixture that contained 10.0 µL of 2x QX200 ddPCR EvaGreen Supermix from Bio-Rad, 1.0 µL of 10 µM of the sense primer, 1.0 µL of 10 µM of the antisense primer, 12.0 µL of dH_2O, and 1.0 µL of the undiluted DNA sample. The genes, primers, and annealing temperatures for these assays are shown in Table 4. The PCR program used for these reactions was: 95 ºC for 15 mins, 40 cycles of 95 ºC for 30 s and 56 ºC for 1 min, then 4 ºC for 5 mins, 90 ºC for 5 mins, and refrigerate at 4 ºC.

Table 4. Target Gene Primers and Sequences for Host-Specific *Bacteroides sp.* ddPCR.

Target	Target Gene	Primer	Sequence (5'-3')	Annealing Temp (°C)	Product	Reference
Ruminant-specific *Bacteriodales*	*BacB2*	BacB2-590F BacB2-708R	ACAGCCCGCGATTGATACTGGTAA CAATCGGAGTTCTTCGTGAT	56	99bp	Mieszkin et al., 2010
Pig-specific *Bacteriodales*	*Pig2Bac*	Pig2Bac-41F Pig2Bac-163R	GCATGAATTTAGCTTGCTAAATTT ACCTCATACGGTATTAATCCGC	56	116bp	Mieszkin et al., 2009
Human-specific *Bacteriodales*	*HF183*	HF183-F HF183-R	ATCATGAGTTCACATGTCCG CTTCCTCTCAGAACCCCTATCC	56	126bp	Green et al., 2014
Human-specific *Bacteriodales*	*HumM2*	HumM2-F HumM2-R	CGTCAGGTTTGTTTCGGTATTG TCATCACGTAACTTATTTATATGCATT	56	101bp	Shanks et al., 2016
Cow-specific *Bacteriodales*	*CowM2*	CowM2-F CowM2-R	CGGCCAAATACTCCTGATCGT GCTTGTTGCGTTCCTTGAGATAAT	56	92bp	Shanks et al., 2008
Cow-specific *Bacteriodales*	*CowM3*	CowM3-F CowM3-R	CCTCTAATGGAAAATGGATGGTATCT CCATACTTCGCCTGCTAATACCTT	56	122bp	Shanks et al., 2008

Data Analysis

The gene concentrations in copies/mL for the collected volume of the sample were calculated from the concentrations found from the ddPCR assays in Microsoft Excel. The concentrations of the target gene copies added to the reaction mixture were found by multiplying the gene concentration with the total reaction volume and dividing by the dilution factor. To find the total amount of copies in the collected sample, the gene concentration added to the reaction was multiplied by the 100 µL of eluted DNA and divided by the total volume from the water sample. These concentration values were log-transformed and plotted against the 10 min resolution data for precipitation, turbidity, and discharge in OriginPro. The loading values in copies/second for the genes were found by multiplying the gene concentration in copies/L by the discharge values. The discharge values utilized corresponded to the sample collection times. The samples that did not have collection times recorded the average discharge for the sample date were utilized.

RESULTS AND DISCUSSION

Environmental Parameters

The changes in the amount of precipitation, the discharge rate of the waterfall, and the turbidity of the water influenced the concentrations of bacteria in the groundwater. These data were collected at the sampling site in intervals of 10 mins over the two-year period. The rainfall data indicate more precipitation in the late spring and summer than autumn and winter; however, there was a large amount of rainfall between September 2015 and September 2016 due to an increased number of rainfall events throughout the year. The amount of water being flushed through the waterfall, or the discharge rate, is dependent upon precipitation, so an increase in rainfall could lead to a larger discharge value. The rainfall and discharge amounts around September 1, 2017, were high because of a rainfall event associated with Hurricane Harvey. The other high values of discharge are due to rainfall events with high precipitation amounts flushing the system. The turbidity of the water is influenced by the rainfall events as well, due to soil and sediment washing in during some events. Various soil, sediment, or waste particles and bacteria adhering to the particles are carried by the rainwater and introduced to the groundwater system. Therefore, a high turbidity value suggests that there will be a high bacterial gene concentration, assuming the bacteria have adhered to the particles being transported in the rainwater, which is seen frequently in WF1 (Figure 3).

Bacterial Concentrations

The log-transformed results from the bacterial target gene concentrations are presented in Figure 3, along with the hydrogeochemical parameters collected in 10 minutes

intervals. The 16S gene was previously found for these samples in qPCR to estimate the total amount of bacterial DNA isolated in this groundwater system (Antle, 2018). As summarized in Table 3, two bacterial species were targeted with ddPCR: *E. coli* (EC) and *Enterococci* (ENT). The EC-specific gene was *uidA*, encoding for the EC beta-glucuronidase that is involved in carbohydrate metabolism (Novel et al., 1973). EC are found in human and animal intestines and would indicate fecal contamination (Denamur et al., 2021). The gene specific to ENT was the ENT 23S ribosomal RNA (rRNA) gene. While ENT can also be found in the intestines of mammals and used to identify fecal waste contamination, these bacteria are also found in the soil, sediments, and plants (Byappanahalli et al., 2012). Therefore, in this setting, ENT would be associated with the environment rather than contamination from fecal waste.

The EC gene concentration was found to be low for this two-year period, as expected. Since EC is mainly found in the mammalian intestines and fecal waste, the concentration of an EC-specific gene would be a better fecal indicator than ENT, as ENT can come from non-fecal sources (Byappanahalli et al., 2012). That is, significant EC gene concentrations would likely indicate contamination of the groundwater with fecal waste. Notably, facilitated by the heavy rainfall events, there were high values of turbidity around January 6, 2016, and April 18, 2017. These conditions likely involved introducing fecal matter to the groundwater system because there were increases in the EC gene concentration of these sample dates; however, because the amount of EC gene was found zero gene copies/mL after these dates, it is likely the case that more fecal contamination did not occur and that the EC did not tolerate the environment. Additionally, due to the

high discharge values on these dates, it is probable that the nutrients supporting growth were flushed out of the waterfall, causing a decrease in the EC concentration.

Unlike the EC results, the results of the ENT-specific gene showed varying concentrations over the two-year period. Interestingly, the amount of the ENT gene found between consecutive weeks increased when discharge was not significantly high, suggesting that the rain could be introducing more ENT from the surrounding soil and plants into the system. Moreover, there is the possibility that ENT was proliferating during these weeks because due to the discharge levels, the metabolites are not being flushed out. Nevertheless, ENT appears to be present in the waterfall throughout most weeks. The weeks where the ENT gene was found to be zero gene copies/mL also had low amounts of 16S, suggesting that these bacteria make up a large portion of the bacterial population in the water.

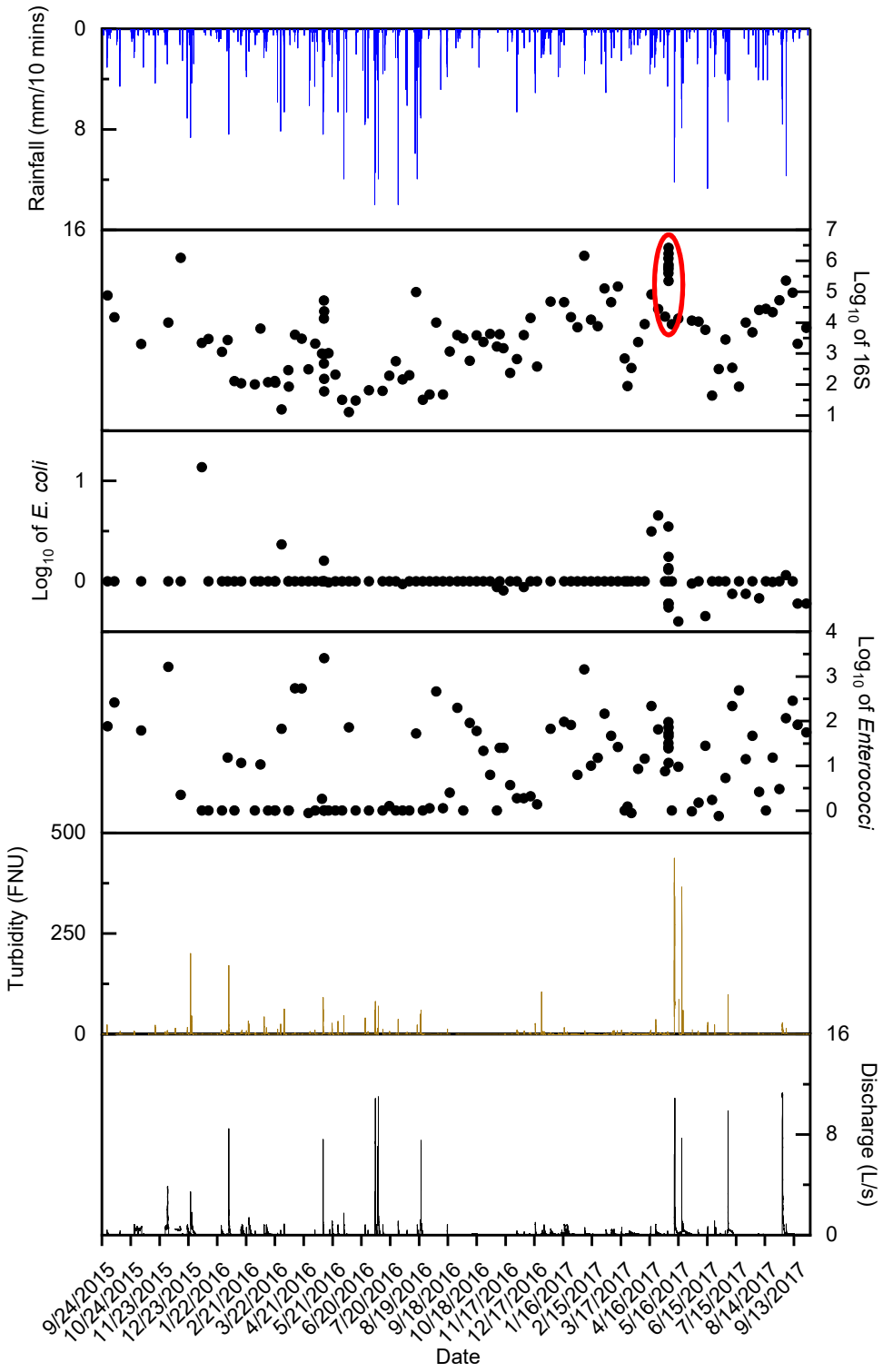

Figure 3. Log-Transformed Bacterial Target Gene Concentrations from Probe ddPCR.

Antibiotic Gene Concentrations

The resulting target gene concentrations from the antibiotic resistant genes (ARGs) ddPCR assays and probe ddPCR assays are shown as a log-transformed heat map in Figure 4. Weeks in which groundwater samples were not collected are noted with the asterisks. The ARGs studied are some of the more abundant ARGs in antibiotic resistant bacteria (ARB). They are common because of the overuse of certain types of antibiotics that led to bacteria becoming resistant. Specifically, bacteria with *tet(A)*, *tet(B)*, or *tet(M)* are resistant to tetracyclines, bacteria with *sul1* or *sul2* are sulfonamide-resistant, and bacteria with *erm(B)* are erythromycin-resistant, as summarized in Table 2 (Zhang et al., 2012; Gupta et al, 2003; Antunes et al., 2005). Tetracycline prevents protein synthesis by binding to the ribosomal 30S and 50S subunits of the bacteria, and sulfonamide targets an enzyme involved in the folate synthesis pathway to prevent bacterial growth and replication. Erythromycin is a type of macrolide that also prevents protein synthesis by binding to the bacterial 50S subunit (Vikesland et al., 2018).

Though these ARGs are abundant, most of the concentrations of the target genes were found to be low, indicating that while there are ARB in this groundwater system, they seem to be present at low amounts. Most ARGs had fluctuations over the two-year collection period, implying more ARB were being introduced. *tet(B)*, *tet(M)*, *erm(B)*, and *sul1* had significant changes in their respective concentrations. Most of their concentration increases, along with those of *tet(A)* and *sul2*, were around the same time as one another, suggesting that the hydrogeochemical parameters were high during these dates and the environmental conditions influence the prevalence of these ARGs.

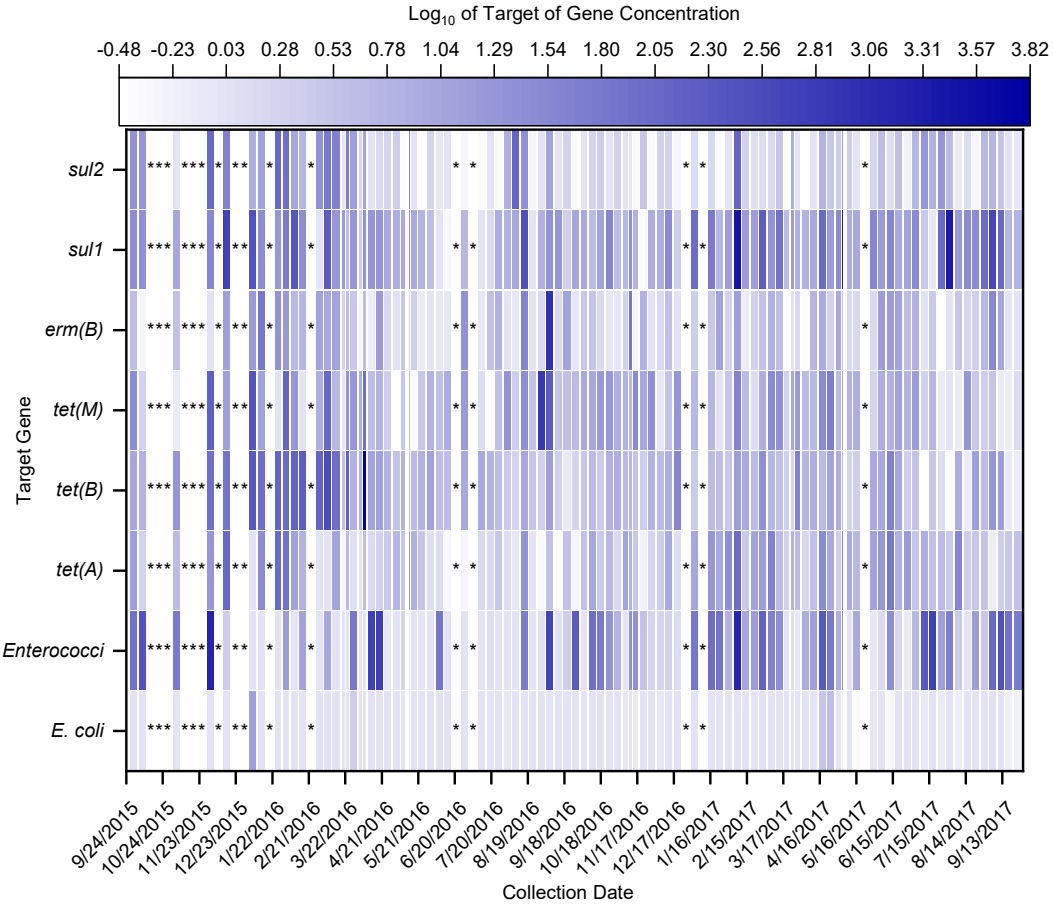

Figure 4. Heat Map of Log-Transformed Target Gene Concentrations from ARG and Probe ddPCR. The asterisks represent the weeks where groundwater samples were not collected over the two-year period.

The ARG concentrations are influenced by the rainfall patterns, levels of turbidity of the groundwater, and the amount of discharge from the waterfall. When there is heavy rain, it is probable that the ARG concentrations in the system will increase, assuming the ARB are already present in the surrounding area. In the autumn of 2015 and winter of 2016, there was a large amount of precipitation in this area, and all the ARG concentrations were high around this time, as seen in Figure 4, suggesting that these genes were being introduced into the system by the rainwater. Additionally, the ARB were likely carried into the groundwater by adhering to particles in soil and fertilizer. This movement is seen in the high turbidity values between late 2015 and early 2016, indicating that foreign materials

from the cave surroundings were being bought into the groundwater by rainwater. However, in these few months of heavy rainfall, there were also high discharge values that would rapidly remove the bacterial nutrients from the system.

Between August and September of 2016, there was another significant increase in most ARG concentrations. This period of two months had high amounts of rainfall and discharge and a relatively high level of turbidity. These data suggest that while a large amount of ARB were likely flushed in initially, the continuous rain that enters the system will cause a dilution to the bacterial gene concentrations. The high discharge value likely corresponds to nutrient flushing as the gene concentrations decreased after this period (McCormack et al., 2016). Similarly, there was an increase between April and May of 2017. This period had substantial rainfall, discharge, and turbidity and also occurred after the typical amendment application period for agriculture in the area. The ARG concentrations returned to approximately the levels they exhibited before the change, likely due to the same reasons.

The log-transformed results for *tet(A)*, *tet(B)*, and *tet(M)* ddPCR are presented in Figure 5 with precipitation amount, discharge level, and turbidity over the study period. The amount of *tet(A)* had some variation between each week. This gene had increases in concentration during December 2015 and January 2016 when there was high rainfall and turbidity. The decrease in *tet(A)* during and after these months, indicating that the high amount of water removed, seen through the discharge rate, flushed nutrients needed for bacteria associated with this gene to proliferate. There were notable increases on February 7 and May 5, 2017. On February 7, 2017, there was relatively high rainfall with low discharge and turbidity values, suggesting that while the introduction of the ARB with

tet(A) may have occurred, the removal of nutrients used by these bacteria through discharge likely did not occur. The amount of *tet(A)* released from the waterfall was calculated to be 15575.6 copies/s, compared to the 101.7 copies/mL found for the WF1. The next week, there was no high rainfall, indicating additional bacteria likely were not introduced, so it is likely the decrease in concentration seen is due to a dilution caused by the rain. On May 5, 2017, there was a significant amount of precipitation that led to high turbidity that brought *tet(A)* to the waterfall. The high discharge likely caused the flushing through the system, leading to decreasing concentration by the following week.

The *tet(B)* did not show much variation between each week; yet, the concentration increased between December 2015 and April 2016, with the highest concentration for *tet(B)* being associated with the sample collected on April 5, 2016. The high gene concentrations during these months are associated with the high amounts of precipitation and turbidity values, indicating the introduction of ARB with *tet(B)* potentially from manure fertilizer. The concentration for *tet(M)* varied greatly. Like the *tet(A)* and *tet(B)*, high amounts of *tet(M)* were found during December 2015 and April 2016 but appeared to decrease by the following week, suggesting that a dilution from the rain may have occurred or that the discharge may have removed nutrients necessary the growth of ARB associated with *tet(M)*. Similarly, between August and September 2016, there was a substantial increase in *tet(M)* that corresponded to a large amount of precipitation during these weeks and relatively high turbidity. These parameters, along with the increase in 16S, indicate that bacteria were introduced. The *tet(M)* concentration decreased by the middle of September 2016 when there was some discharge recorded, suggesting that nutrients for bacterial growth were flushed out of the groundwater system.

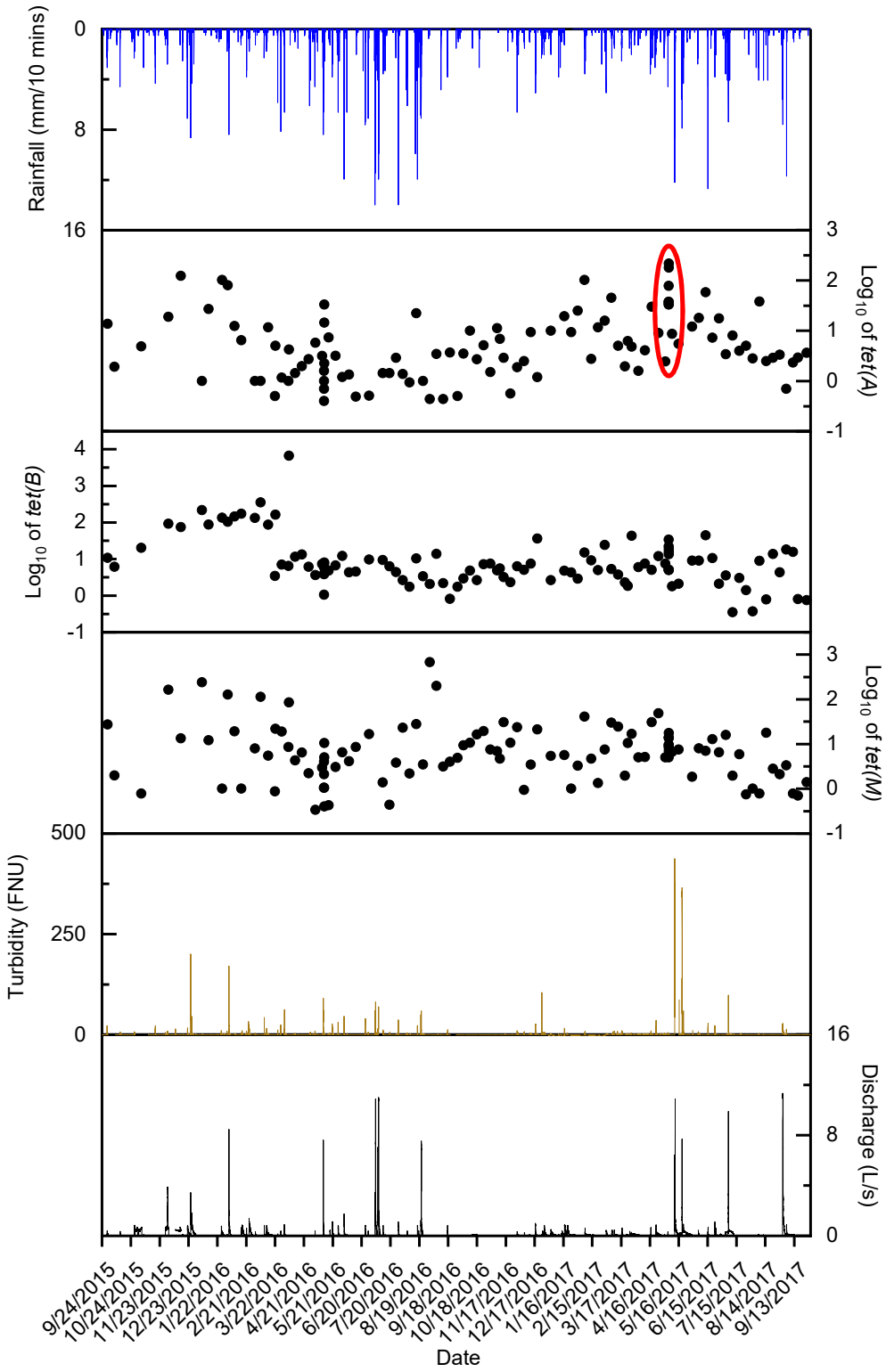

Figure 5. Log-Transformed of *tet(A)*, *tet(B)*, and *tet(M)* Gene Concentrations from ARG ddPCR.

The log-transformed results for *erm(B)*, *sul1*, and *sul2* ddPCR are presented in Figure 6 with rainfall, turbidity, and discharge rate over the study period. The quantity of these genes in Figure 6 had noticeable variation. Interestingly, these concentrations were found to be relatively high in 2015 and decrease until about September 2016; then, they slowly increase after that time. This overall pattern was likely due to the lower rainfall recorded between September 2016 - September 2017 that would not be able to introduce as many ARB into the water, further showing how these hydrogeochemical parameters seem to influence the ARG amounts in the groundwater system.

Like the previous three ARGs, the amounts of *erm(B)*, *sul1*, and *sul2* were found to be relatively high between December 2015 and April 2016, due to the heavy precipitation and high turbidity recorded. These values decreased when high discharge values were recorded. After April 2016, the *erm(B)* concentration did not seem to vary as much as it did before that period, but this lack of variation can be attributed to the relatively low levels of turbidity, which indicate that foreign particles that potentially have bacteria adhered to them were not being introduced into the system as readily; yet, there were still increases, notably a large amount of *erm(B)* found in the sample collected on September 6, 2016, which was also found to be high for *tet(M)*. Therefore, like *tet(M)*, the concentration of *erm(B)* decreased by the following week when a relatively high discharge was recorded, indicating that nutrients were flushed out.

The concentration of *sul1* quantified varied significantly while *sul2* did not differ as much. Both genes were seen to increase in the middle of August 2016, which had heavy rainfall and relatively high turbidity, but by the end of that month, these gene concentrations were found to be very low or 0 copies/mL, which corresponded to the high

discharge values during the latter half of the month. Additionally, on February 7, 2017, there were noteworthy increases in the concentrations of *sul1* and *sul2*, along with *tet(A)*. Both the turbidity and discharge values recorded for this period were relatively low, suggesting that the number of contaminant particles was low and that the nutrients would likely not be flushed out, respectively. Yet, because of the relatively high amount of rainfall, the ENT and 16S concentrations had notable increases while EC did not change, indicating that bacteria not associated with fecal waste entered the groundwater system. The amounts of *sul1* and *sul2* had substantially decreased by the following week. The amount of *sul1* increased between August and September 2017 because of the heavy rainfall associated with Hurricane Harvey, and due to a large amount of precipitation, the turbidity was relatively high, potentially increasing the amount of *sul1*; yet, the discharge values were also high, contributing to the decrease in *sul1*.

The high-resolution sampling event on May 5, 2017, saw increases in the concentrations of bacteria and many genes, specifically the 16S gene, *tet(A)*, *sul1*, and *sul2*. These increases are explicitly shown by the red ovals in Figures 3, 5, and 6 and indicate how the concentrations are affected by the storm events. Due to the heavy rainfall and turbidity values recorded for this day, there was an initial pulse of these genes. Then, it seems that the continuous rain entering the system led to dilutions of these gene concentrations, seen in their respective decreases after the initial pulse.

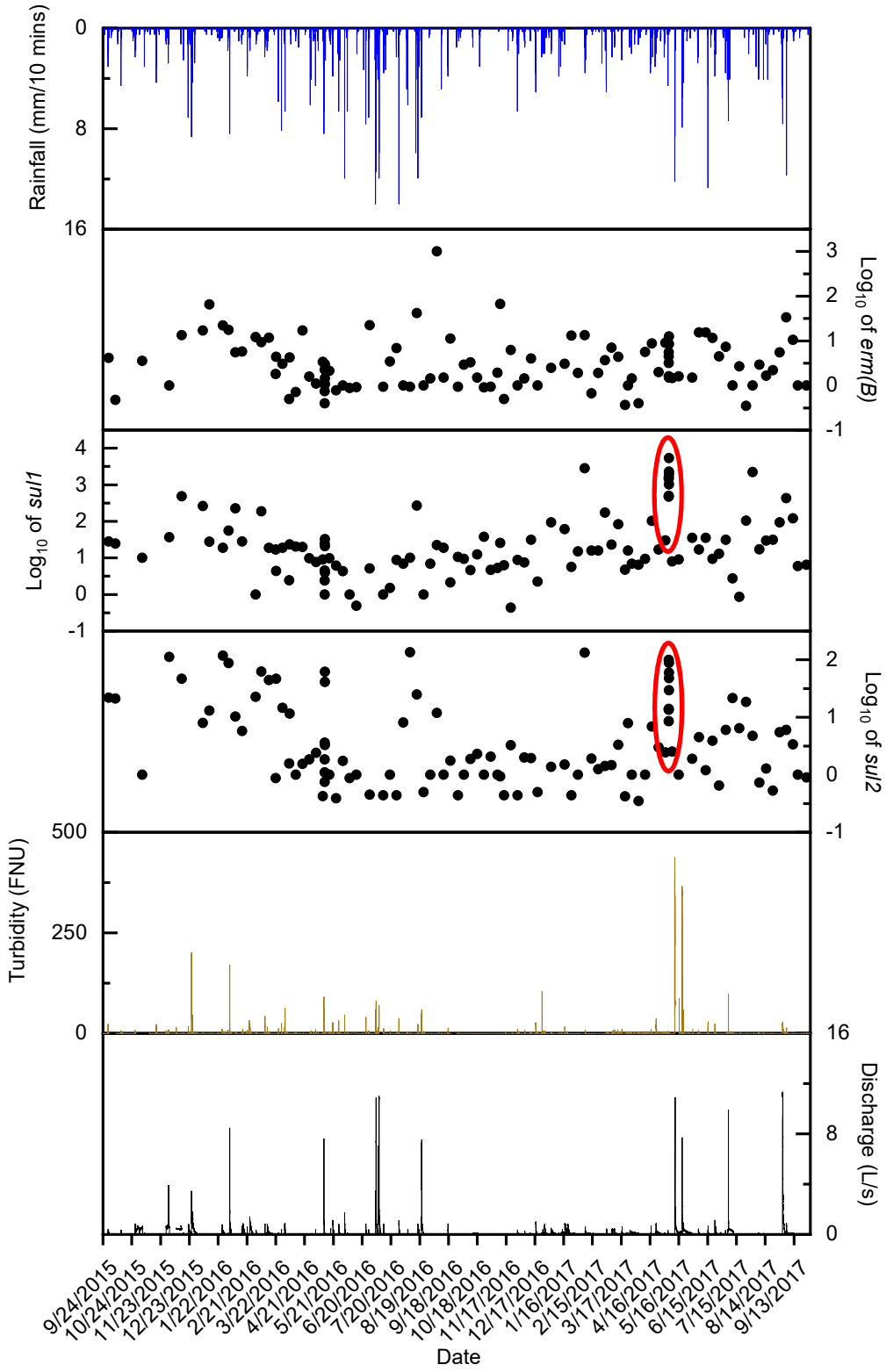

Figure 6. Log-Transformed of *erm(B)*, *sul1*, and *sul2* Gene Concentrations from ARG ddPCR.

Most of the genes targeted in the endpoint PCR assays were absent. Figure 7 shows these results with the only positive band being present for *bla*$_{CTX\text{-}M}$; this positive band in Figure 8A corresponds to the sample collected on February 7, 2017. Figure 8B shows positive controls utilized for both *bla*$_{CTX\text{-}M}$ and *bla*$_{CMY\text{-}2}$. The target genes for these endpoint PCR assays, shown in Table 1, are associated with EC (Vikram et al., 2017; Chen et al., 2012). Therefore, it is unsurprising that these genes were not present in the groundwater, because the EC concentrations over the sampling period were very low. The positive *bla*$_{CTX\text{-}M}$ on February 7, 2017, may have been from other non-EC bacterial sources because the EC gene concentration was found to be zero copies/mL for that day, but the 16S gene concentration was 1,440,000 copies/mL.

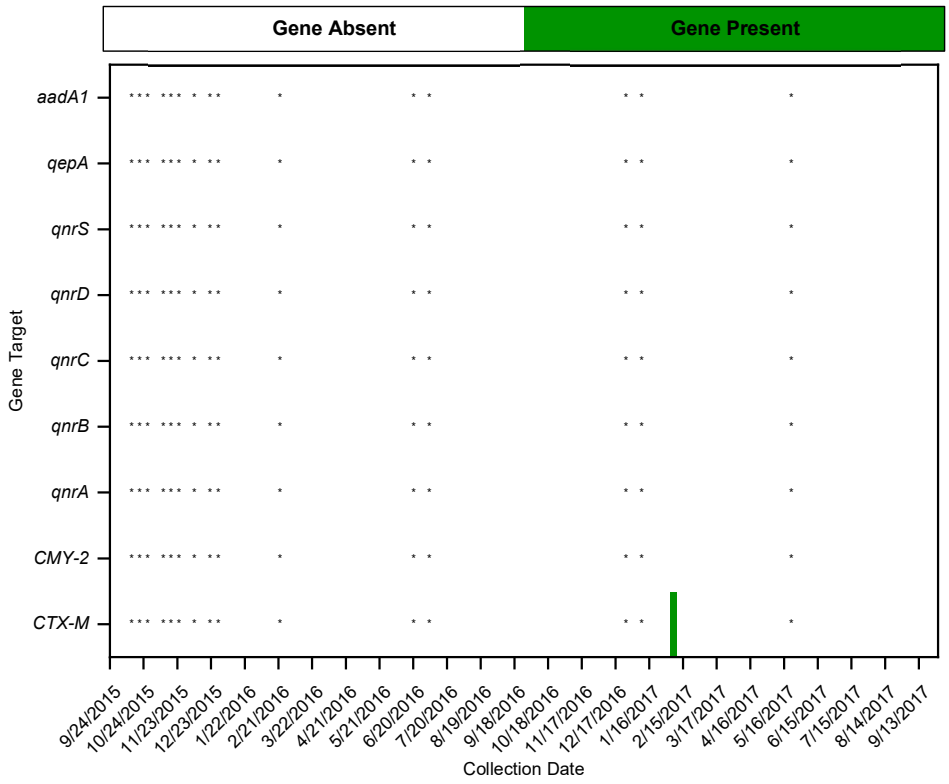

Figure 7. Presence of ARGs in Endpoint PCR.
The asterisks represent the weeks where groundwater samples were not collected over the two-year period.

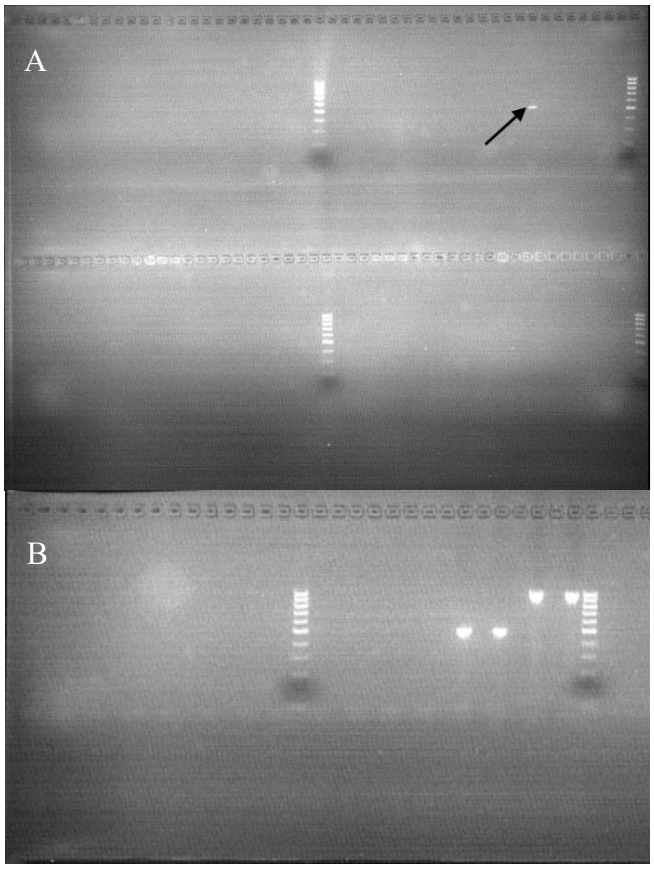

Figure 8. Gels for *bla*$_{CTX-M}$ and *bla*$_{CMY-2}$ Endpoint PCR

Host DNA Markers

The log-transformed gene concentrations of the host-specific *Bacteroides sp.* ddPCR assays are shown in Figure 9. These assays targeted genes specific to the digestive system of animals and humans to determine whether fecal contamination from certain sources occurred. As summarized in Table 2, the genes utilized targeting pig (*Pig2Bac*), cow (*CowM2* and *CowM3*), and human (*HumM2* and *HF183*) *Bacteroides*, as well as a general *Bacteroides* for herbivorous mammals (*BacB2*). Fertilizer products are applied to corn and soybean crops on the land above the cave. This application of animal waste as fertilizer was previously reported to occur in late December and February, with crops being planted in April, but antibiotic resistant gene presence in this waste is unknown (Vanderhoff, 2011; Antle, 2018). Depending on the rainfall patterns, manure may be

introduced into the groundwater, leading to ARB and ARGs becoming present in the water. Importantly, the surrounding area above the cave does not have cattle or swine farms, indicating that other than the fertilizer and human waste through leakage, there is no other natural method of introducing ARGs to the system.

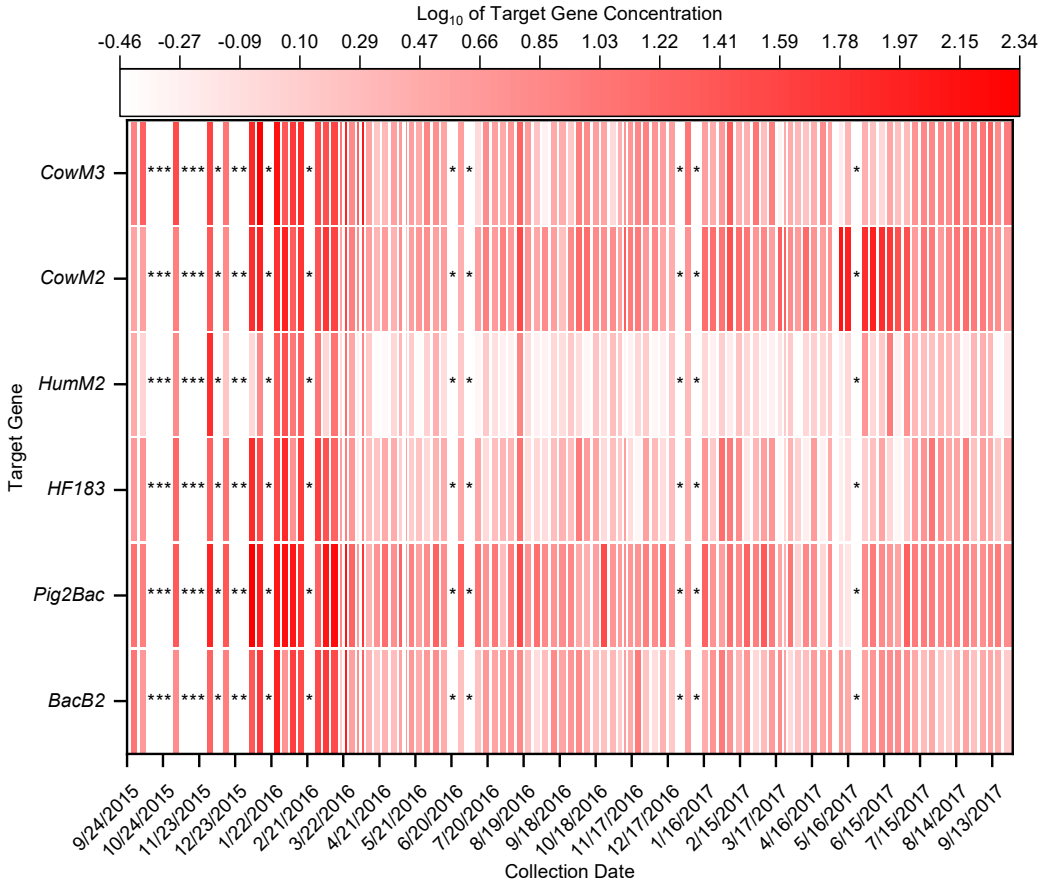

Figure 9. Heat Map of Log-Transformed Host Specific *Bacteroides sp.* ddPCR Gene Concentrations. The asterisks represent the weeks where groundwater samples were not collected over the two-year period.

During the heavy rainfall events between September 2015 and September 2016, the gene concentrations of host DNA targets were high between the fertilizer application in December and the crop planting in April. The introduction of the mammalian fecal waste to the system also explains the increase in the EC gene concentration on January 2016 and April 2017, as fecal waste contains EC. In both cases, it is probable that a mixture of soil

and manure was introduced into the groundwater system by the rain because the turbidity values for these dates are high, indicating foreign particles entered the waterfall.

The log-transformed results for *BacB2* and *Pig2Bac* ddPCR are presented in Figure 10 with rainfall, turbidity, and discharge rate over the study period. The quantities of these genes do not appear to have much variation between April and December. Because fertilizer was applied in December, the heavy rain that was seen between the 2015 and 2016 transition likely washed the manure into the waterfall, as seen from the turbidity increase during this period. While high discharge values were recorded during this period, the effect on the concentrations seemed to be minimal. *BacB2* was found to decrease likely because of a lack of metabolites removed by the high discharge in late January, but the *Pig2Bac* concentration did not have a substantial increase. This difference suggests that more of the bacteria associated with *Pig2Bac* were being introduced into the water, whereas those associated with *BacB2* were not because the turbidity was also recorded to be relatively high around this date, suggesting that more fertilizer was washed into the system. The amount of these genes remained relatively high in the water until about April 2016 when there was relatively high precipitation and discharge. Due to there being less rain between 2016 and 2017, notable increases in these host DNA markers are not seen because the bacteria have no means of entering the groundwater system.

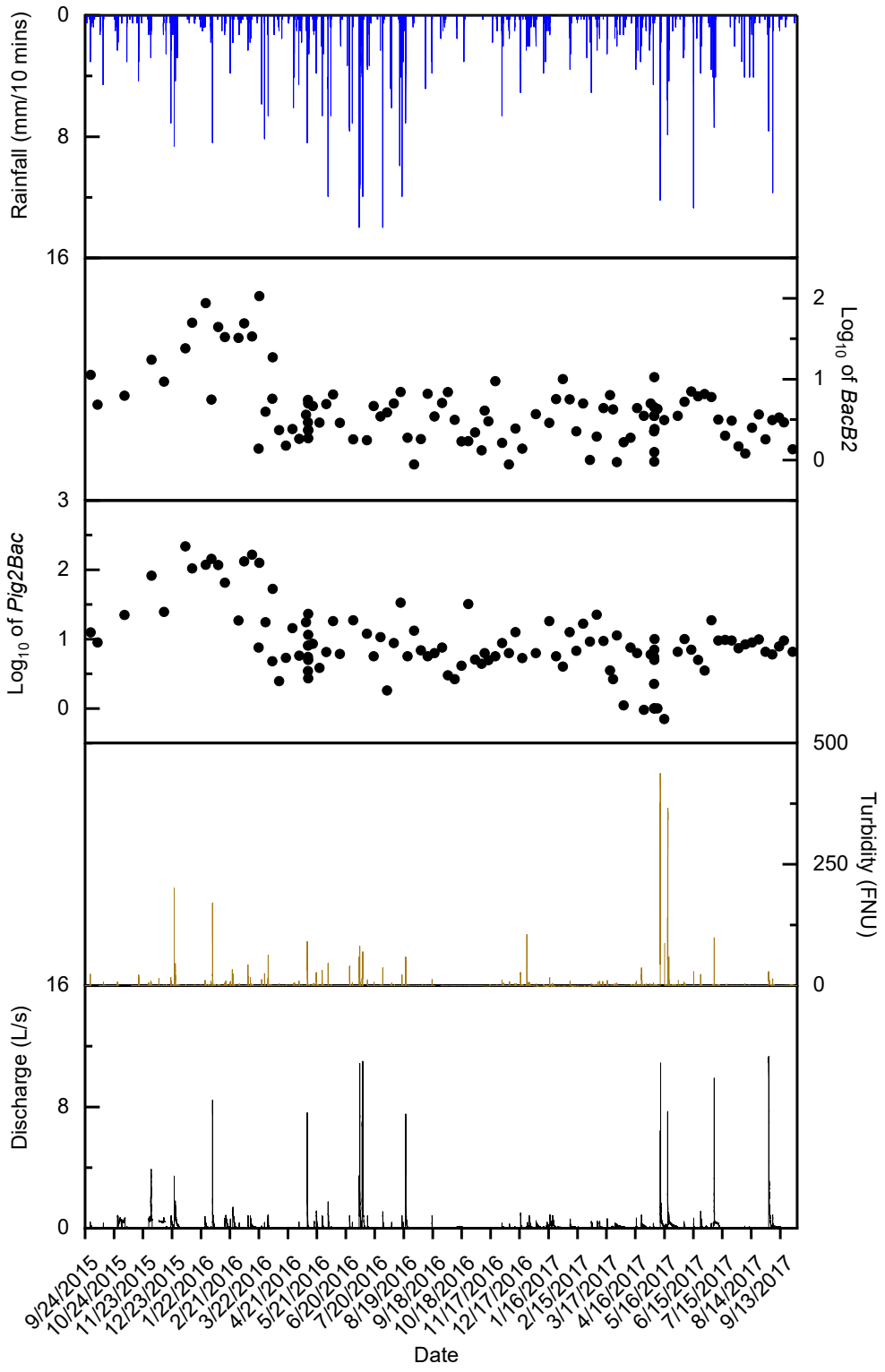

Figure 10. Log-Transformed Gene Concentrations of *BacB2* and *Pig2Bac* ddPCR.

The log-transformed results for *CowM2* and *CowM3* ddPCR are presented in Figure 11 illustrating rainfall, turbidity, and discharge rate over the study period. While the concentration of *CowM3* seems to not change much, the amount of *CowM2* varies greatly. Like the other genes targeted, these genes had increases in their respective concentrations between December 2015 and April 2016. These increases were facilitated by the application of fertilizer in late December, coupled with the high amounts of precipitation during 2015-2016. By the middle of April, the amounts of *CowM2* and *CowM3* had progressively decreased due to the dilutions associated with the heavy rainfall. Additionally, the quantity of *CowM2* had notable changes in May and June of 2017. This period recorded high precipitation and turbidity values, indicating that movement of particles by rainwater was occurring. Interestingly, even with the relatively high discharge values recorded during these months, the concentration of this gene did not decrease much until approximately the end of June 2017. Moreover, the contents of the fertilizer likely contained a mixture of cow and pig fecal waste because their respective *Bacteroides sp.* had significant increases around the times the manure entered the groundwater system.

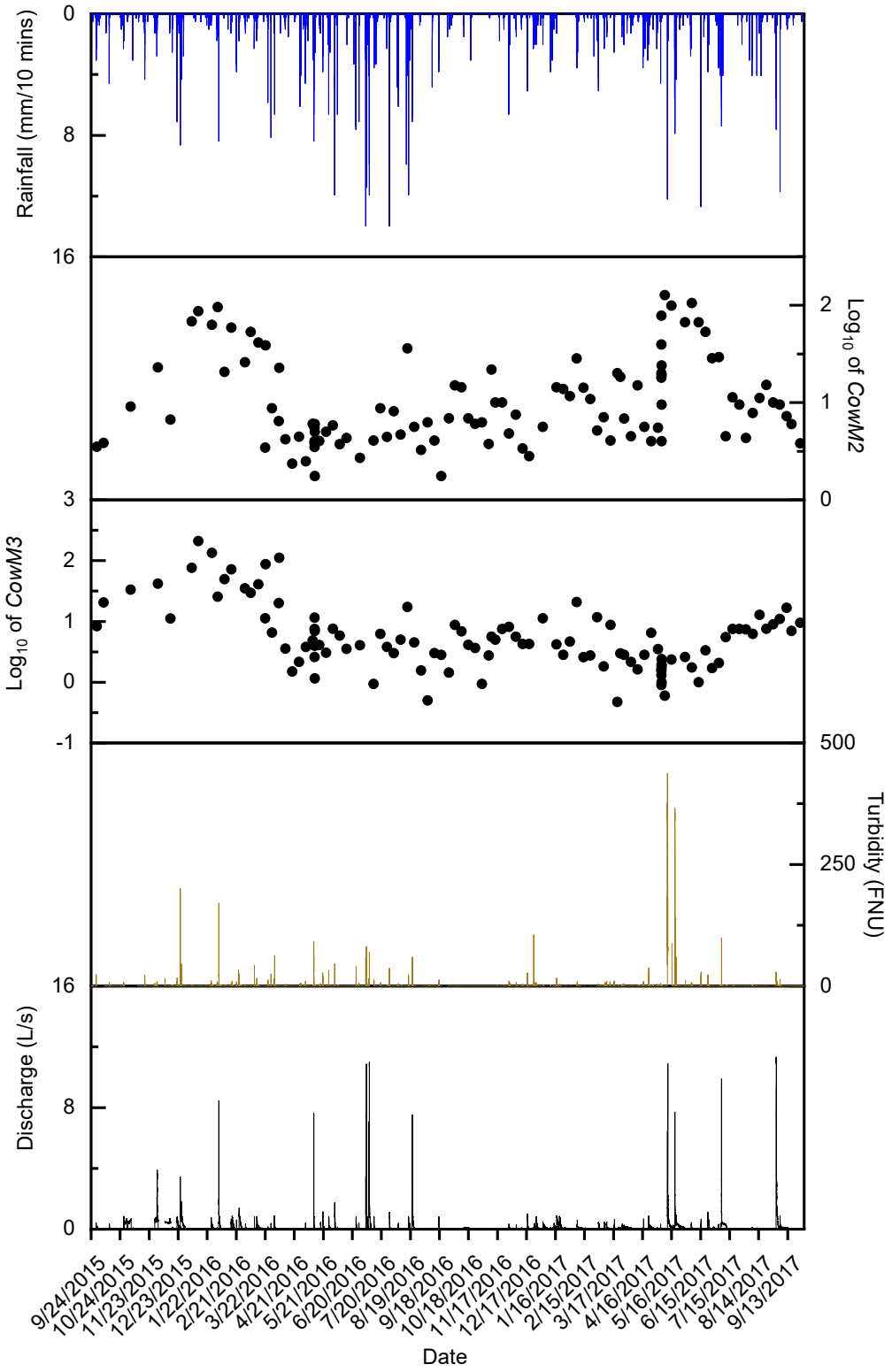

Figure 11. Log-Transformed Gene Concentrations of Cow DNA Markers ddPCR.

The log-transformed results for *HumM2* and *HF183* ddPCR are presented in Figure 12 with rainfall, turbidity, and discharge rate over the study period. The quantity of these genes appears to have some variation between samples and one another. Expectedly, these genes were found to be less than 100 copies/mL, indicating that contamination of human fecal waste did not occur at a notable level; yet, like the other target genes, there were relatively high values between December 2015 and April 2016. These increases may be attributed to the overflowing of septic tanks in the few houses near the cave from the high amounts of rain seen during this time. Additionally, the human DNA markers would have no other method of introduction into the groundwater other than from these septic tanks.

Interestingly, the host DNA target genes with the same host lead to different results between the two genes. *CowM2* and *CowM3*, both targeting cow-specific *Bacteriodales*, have some differing gene concentrations, as seen in Figure 9. These differences were likely due to the higher sensitivity and specificity of *CowM3* compared to that of *CowM2* (Xue et al., 2019). *HumM2* and *HF183* exhibit slight concentration differences but not to the same degree as the cow DNA markers. The differences between the human DNA markers may be due to a lower specificity of *HumM2* caused by the different fecal sources present in the water (Green et al., 2014). Nevertheless, there appears to be some human waste present in the water system, potentially caused by leakage from septic tanks.

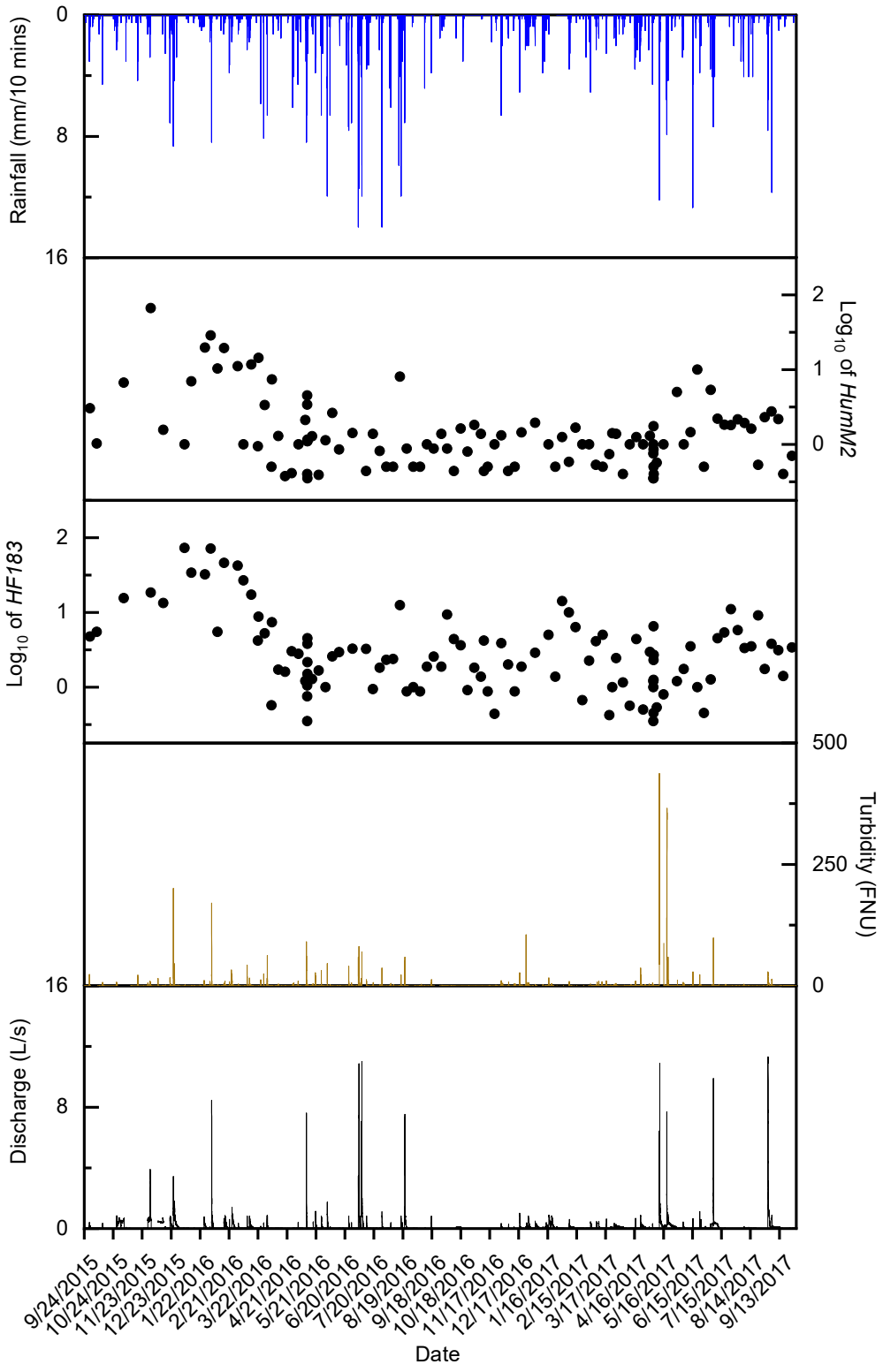

Figure 12. Log-Transformed Gene Concentrations of Human DNA Markers ddPCR.

CONCLUSION

While ARGs do seem to be present in the groundwater system, their respective concentrations vary with environmental factors, such as rainfall, waterfall discharge, and groundwater turbidity. The rainwater appears to carry the ARB attached to external particles into the groundwater, but while the discharge may flush out nutrients for the ARB proliferation, that is not yet clear. However, the ARGs must be present in the environment before being introduced to the groundwater system by precipitation. Additionally, the timing of fertilizer application seems to influence the ARG concentrations because the gene concentrations appear to increase after the fertilizer has been applied, assuming that there is enough rainfall to transport the ARB to the groundwater. Whether new ARGs are being introduced by the fertilizer, or if the ARB already in the system are proliferating, is still unclear from this study. Residence time and longevity of the ARB to persist in the karst groundwater system is still unknown but may play a factor in the prevalence and persistence of ARB over longer periods of time.

In agricultural areas underlain by karst aquifers, groundwater quality seems to be vulnerable to contamination from amendment application both due to infiltration of the waste and due to the prevalence of ARB in the environment. From this case study, it appears the soil-rock interface is an important location from which these bacteria can be flushed into the broader aquifer system during storm events. Thus, improved best management practices should consider the timing and amount of amendment application on cropland and additional remediation techniques, like the use of biochar and cover crops.

Future studies should include examining other factors, such as nutrient flux and initial concentrations of bacteria and ARGs in amendments. Studying the nutrient flux may elucidate if the bacteria are using these nutrients for proliferation and if the varying discharge of the system affects the ARG concentrations. Determining the concentrations of these bacteria and ARGs in applied animal manure could indicate if the ARB are already present in the organic fertilizer or if they evolving, or acquiring resistance, during their time in the groundwater system and how long their DNA is surviving in the environment. It may be the case that these bacteria are dormant, or non-dividing and metabolically inactive, but they harbor ARGs and can remain in the groundwater. It has been previously found that for some nonproliferating bacteria, they can survive up to 100 days in the groundwater (Filip et al., 1988). Thus, more in-depth studies to determine if these ARB proliferate from the source or within the groundwater system, or perhaps both, should be conducted in order to better understand ARB presence in karst groundwater systems.

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