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Environmental Significance

Fecal contamination of surface waters in karst terrains poses a significant threat to human health, as pathogens introduced through fecal sources can rapidly enter groundwaters that are often used for drinking purposes. Additionally, environmental processes and land management practices can further aggravate the contamination. Here, we demonstrate that a better understanding of nutrient loading and fecal contamination of water sources to implement best management practices can be achieved when physico-chemical and microbial source tracking data is combined with environmental processes (precipitation) and land use/management practices (septic tank density) data. Furthermore, spatial clustering results generated in this study provide cost-effective solutions by prioritizing the sampling sites for fecal pollution monitoring.

Fecal pollution source characterization in the surface waters of recharge and contributing zones of a karst aquifer using general and host-associated fecal genetic markers

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Abstract

Fecal pollution of surface waters in karst-dominated Edwards aquifer is a serious concern as contaminated waters can rapidly transmit to groundwaters, which are used for domestic purposes. Although microbial source tracking (MST) detects sources of fecal pollution, integrating data related to environmental processes (precipitation) and land management practices (septic tanks) with MST can provide better understanding of fecal contamination fluxes to implement effective mitigation strategies. Here, we investigated fecal sources and their spatial origins at recharge and contributing zones of Edwards aquifer and identified their relationship with nutrients in different environmental/land-use conditions. During March 2019 to March 2020, water samples (n=295) were collected biweekly from 11 sampling sites across four creeks and analyzed for six physico-chemical parameters and ten fecal indicator bacteria (FIB) and MST-based qPCR assays targeting general (*E. coli*, *Enterococcus*, and universal *Bacteroidales*), human (BacHum and HF183), ruminant (Rum2Bac), cattle (BacCow), canine (BacCan), and avian (Chicken/Duck-Bac and GFD) fecal markers. Among physico-chemical parameters, nitrate-N (NO_3-N) concentrations at several sites were higher than estimated national background concentrations for streams. General fecal markers were detected in majority of water samples, and among host-associated MST markers, GFD, BacCow, and Rum2Bac were more frequently detected than BacCan, BacHum, and HF183, indicating avian and ruminant fecal contamination is a major concern. Cluster analysis results indicated that sampling sites clustered based on precipitation and septic tank density showed significant correlation (p <0.05) between nutrients and FIB/MST markers, indicating these factors are influencing the spatial and temporal variations of fecal sources. Overall, results emphasize that integration of environmental/land-use data with MST is crucial for a better understanding of nutrient loading and fecal contamination.

1. Introduction:

Karst terrains constitute around 10% of the land surface on earth and approximately 25% of the world's population relies on vulnerable water resources from karst aquifers for drinking, agriculture, and industrial needs.[1, 2] In the USA, around 20% of the land surface is categorized as karst terrain and about 40% of the groundwater supplies for domestic purpose comes from karst aquifers.[3] However, karst aquifers are extremely susceptible to contamination as large voids and conduits, which are characteristics of this system, can facilitate rapid transport of surface waters to the subsurface.[4] Contaminated surface waters in the recharge areas of karst aquifers can be rapidly transmitted to groundwater sources with little or no filtration.[5] Fecal contamination of surface water resources in such settings may lead to water-borne disease outbreaks and economic losses.[6] Previous reports indicated that 26% of water-borne disease outbreaks for groundwater sources in the USA are due to the karst topography factor.[7] Therefore, effective control and estimation of risk associated with fecal contamination of surface waters in the karst aquifer region are essential to take proper mitigation efforts by the water management authorities to prevent human health risks.

Fecal contamination of environmental waters can originate from human and animal waste sources and determining the source of fecal contamination is crucial for implementing remedial actions. Potential human waste sources include effluent from the community's wastewater treatment system and on-site sanitation (septic) systems, while animal waste sources include domestic and wild animals, discharge from livestock waste pits or lagoons, and manure applied to agricultural farms.[8] As human and animal fecal contamination of environmental waters can increase the occurrence of pathogens, traditional fecal pollution monitoring methods rely on the enumeration of fecal indicator bacteria (FIB) to assess the microbiological water quality and

associated public health risks.[9] However, there are several limitations to these traditional fecal monitoring methods; for instance, they do not determine the source or origin of fecal contamination and have poor correlation with the presence of pathogens.[10, 11] In this regard, microbial source tracking (MST) techniques have been developed to identify fecal contamination sources in the environment. Several culture-based and molecular-dependent MST methods were proposed to differentiate the human and animal sources of fecal contamination in the environment.[12-14] Among these, quantitative PCR (qPCR) based MST methods targeting host-associated bacterial, viral, or mitochondrial genetic markers have been mainly used to quantify the sources of fecal pollution.[15-21] Overall, bacterial genetic markers targeting host-associated *Bacteroidales* 16S rRNA fragments are more frequently applied for MST studies as *Bacteroidales* are obligate anaerobic bacteria found in the human and animal gut at high concentrations and have limited persistence in the environment.[16, 20, 22, 23] However, avian fecal sources were found to have lower concentrations of *Bacteroidales* and could be identified well by targeting other bacterial taxonomic groups such as *Helicobacter* spp.[24] Consequently, studies applying MST approach to track fecal sources in the surface and ground waters of karst regions have been carried out around the world; although they are less frequent.[6, 8, 25] Moreover, such studies to monitor the sources of fecal contamination in surface waters of the karst-dominated aquifers in the USA are very limited.[8, 26]

While MST studies can identify the sources of fecal pollution, environmental factors can significantly influence the spatial and temporal variation of fecal contamination and cause non-point sources of pollution; for instance, the rate and timing of precipitation and land use of the watershed area can significantly impact the bacterial contamination patterns in rivers and streams.[27, 28] Therefore, studying the impact of environmental and land management practices on fecal contamination is crucial to understand the relationship between microbes and nutrient

contaminants detected in the watershed and also for identifying the effect of non-point sources of pollution on water quality.[29] Although previous studies have examined the relationship between water quality parameters and land use on various scales, limited studies had incorporated the environmental processes and land management practices data with MST, particularly in karst terrains.[29, 30]

The karst-dominated Edwards aquifer in south-central Texas is one of the most permeable and productive aquifers in the United States. As a sole-source aquifer, it provides drinking water source to over two million people and also delivers most of the water required for agricultural and industrial needs in the area.[3] The Edwards aquifer region can rapidly get recharged with surface waters and storm runoffs due to the presence of large voids and sinkholes in the recharge zone, signifying its vulnerability to contamination.[31] Several studies conducted on Edwards aquifer water quality have documented the nutrient contamination of water sources from anthropogenic agents.[31, 32] However, studies focusing on microbial water quality in the Edwards aquifer region are limited.[26] Furthermore, limited studies were conducted so far on understanding the relationship between nutrients and fecal markers in different environmental processes and land management practices of Edwards aquifer. In this regard, we investigated the physico-chemical characteristics and abundance of FIB, general, and host-associated MST fecal markers in the surface water samples collected from four different creeks that flow in the recharge and contributing zones of the Edwards aquifer and explored the impact of environmental/land use characteristics such as precipitation and septic tank density on fecal contamination and nutrient loading. The main objectives of the current study are as follows: (1) to assess physico-chemical characteristics and examine the prevalence and abundance of general and host-associated fecal markers in surface waters collected from the four creeks to identify the contamination source and spatial origins, (2)

to determine the impact of environmental processes (precipitation) and land management practices on the relationship of fecal markers and nutrient contaminants observed, and (3) evaluate the overall implications of physicochemical and microbial water quality of surface waters in the Edwards aquifer region. The results generated in the current study will not only benefit in improving the water quality of the Edwards aquifer region but could be valuable for the advancement of water quality management at other karst aquifer regions by providing useful information related to the implementation of best management practices (BMP), land use management, and in designing monitoring programs.

2. Materials and Methods

2.1. Study area, sample collection, and processing

This study was carried out at four creeks including Leon, Balcones, San Geronimo, and Helotes Creeks that flow in the Edwards aquifer region in Bexar County, Texas. Leon and Balcones Creeks flow through the recharge and contributing zones of the Edwards aquifer, while San Geronimo and Helotes Creeks primarily flow in the contributing zone and reaches the recharge zone only during periods of significant surface runoff and flow.[33] Leon Creek originates on the west side of the city of San Antonio in Bexar County and flows to the south of the city, spanning around 72 km in length and draining more than 500 km^2 of land.[34] The Balcones Creek originates in the southwest side of Bandera County, which is around 1.6 km away from the junction between Kendall, Bexar, and Bandera Counties region. This Creek flows approximately 24 km to the east in the rural areas with light ranch and recreational activities and finally converges with the Upper Cibolo Creek at the Bexar, Kendall, and Comal Counties junction.[26] Previous studies indicated that Leon and Balcones Creeks are facing water quality issues due to fecal contamination.[26, 35] San Geronimo Creek originates in the northwestern region of Bexar County and runs southwest through Bexar,

Bandera, and Medina Counties for about 32 km before converging with Medina River and covers approximately 177 km^2 of the drainage area.[36] Helotes Creek is a relatively small stream that flows on the west side of Bexar County with a stream length of 24 km approximately.[37] Studies focusing on the water quality of San Geronimo and Helotes Creeks are limited[31] and, to our knowledge, field studies to evaluate the fecal contamination of waters in these two Creeks were not carried out previously.

In total, eleven sampling sites were selected for this study including four sites each from Leon (L-1 to L-4) and Balcones Creeks (B-1 to B-4), two sites from San Geronimo Creek (S-1 and S-2), and one from Helotes Creek (H-1) (Fig. 1). The land-use information and geographical coordinates of sampling sites are given in Supplementary Table S1. The sampling events were carried out biweekly during March 2019 to March 2020, and 1-liter water samples were collected from each sampling site using sterile polypropylene bottles (Nalgene, Rochester, NY) and transferred on ice to the laboratory at the University of Texas at San Antonio (UTSA, San Antonio, TX). Water samples (300 mL) were filtered through 0.45-μm-pore-size polycarbonate membranes (Pall Corporation, Ann Arbor, Michigan) and stored at −20 °C until DNA extraction. To check the cross-contamination during sample processing, sterile deionized water was used as a control and was filtered during each sampling event. All the water samples were processed within 24 h for physico-chemical and molecular analysis.

2.2. Physico-chemical analysis of water samples

The United States Environmental Protection Agency (USEPA) regulates the water quality of rivers, lakes, and reservoirs and formulated acceptable maximum contaminant levels (MCL) to monitor the surface water quality.[38] For the current study, the results of physico-chemical analysis of water samples were compared to the acceptable MCLs for streams and rivers as cited by USEPA

and Texas Commission on Environmental Quality (TCEQ). A total of 6 physico-chemical parameters including pH, dissolved oxygen (DO), water temperature (WT), nitrate (NO_3-N), nitrite (NO_2-N), and ammonia nitrogen (NH_4-N), were analyzed as described previously.[26] WT, pH, and DO were measured on-site using Intellical™ LDO101 Field Luminescent/Optical probe and HQ40d portable multi-meter (HACH, Loveland, CO). The analysis of NH_4-N, NO_2-N, and NO_3-N concentrations in the surface water samples was carried out at the UTSA laboratory using USEPA Salicylate Method 10205 (HACH TNTplus 830 ultra-low range kit), Diazotization Method 10207 (HACH TNTplus 839 low range kit) and Dimethylphenol Method 10206 (HACH TNTplus 835 low range kit), respectively. The concentrations were reported as "0" if values were below the detection limits (BDL) as suggested by the manufacturer.

2.3 Genomic DNA extraction and qPCR assays

Genomic DNA was extracted from the filters using the Qiagen DNeasy PowerLyzer PowerSoil Kit (Qiagen; Germantown, MD) by following the manufacturer's instructions. Extraction blanks were included for each batch of DNA extraction to ensure no carryover contamination occurred. DNA concentration and purity of extracts were determined using a Nanodrop Spectrophotometer (Thermo Scientific, Wilmington, DE) and all the DNA samples were stored at −80 °C until further molecular analysis.

A total of ten qPCR assays were used to identify the fecal contamination at the Edwards aquifer. DNA extracted from surface water samples were analyzed for the following FIB and MST qPCR markers using published assays and conditions (Table S2): *E. coli* (EC23S857), *Enterococcus* (Entero1), universal *Bacteroidales* (BacUni), human-associated *Bacteroidales* (HF183, BacHum), ruminant-associated *Bacteroidales* (Rum2Bac), cattle-associated *Bacteroidales* (BacCow), canine-associated *Bacteroidales* (BacCan) and avian-associated fecal

markers (Chicken/Duck-Bac and GFD). All the qPCR assays were carried out using CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA) and all qPCR reactions were performed with 25µL as reaction volume. Except for the GFD assay, all the remaining assays were probe-based and each qPCR reaction mixture (25 µL) contained 12.5µl of iTaq™ Universal Probes Supermix (Bio-Rad, Hercules, CA), 800nM each of respective forward and reverse primers, 100nM of the respective probe and 2 µL of template DNA. For the GFD assay, the qPCR reaction mixture (25µL) included 12.5 µL of SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA), 200 nM each of forward and reverse primers and 2µL of template DNA. The qPCR amplifications were performed with an initial denaturation at 95 °C for 2 min, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C (except Entero1 and GFD, which were performed at 54 °C and 57 °C respectively). For the GFD assay, the melting curve analysis (temperature increases at 60 °C to 95 °C at around 0.4 °C per minute) was carried out after qPCR amplification to validate the specificity of amplified products, and samples were considered positive when the melting points were matched with the qPCR standards melting point within a tolerance of 0.5 °C.[39] Plasmids containing the target sequence for each assay were purchased from Integrated DNA Technologies (IDT, Skokie, IL) and were used as qPCR standards in this study. All the samples, standards, and negative controls were tested in duplicate for each assay, and quantities were determined based on the standard curve generated using serially diluted plasmid standards (10^6 to 10^1 copies/reaction). The absolute gene copies of the markers were calculated as the average concentration of duplicate reactions and reported as Log_{10} gene copies per 100 mL of water.

2.4 Quality control and qPCR data analysis

Controls used for sample processing and DNA extraction, and no-template controls included during qPCR amplifications were analyzed to check cross-contamination. Using BacUni assay, the

DNA extracts were evaluated for the absence of PCR inhibitors by testing at two different dilutions (undiluted and 1:10), and the DNA extract was considered as PCR inhibitors free if both the dilutions gave matching concentrations of Bac-Uni markers [40, 41]. The results of all qPCR assays were processed based on the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines.[42] The details of the limit of detection (LOD), limit of quantification (LOQ), R^2 values, and amplification efficiencies of all the qPCR assays are provided in Supplementary Information. Samples that were below the LOQ and above the LOD levels were considered detected but not quantifiable (DNQ) and samples with below LOD concentrations were considered negative.[41, 43]

2.5 Precipitation and land use data

Precipitation received at each sampling site within 7 days before the sampling event was retrieved from the USGS National Water Information System and is presented in Supplementary Table S3. We explored land use features such as septic tank density, human population, and percent of impervious surface at 1km spatial scales of each sampling site, as fecal marker correlation with land use supports recent contamination from nearby inputs.[26] The 1-km buffer zone around each sampling site was created in ArcMap 10.5.1 version (Environmental Systems Research Institute, Redlands, CA) using the Intersecting Layers Mask tool with sampling site-specific catchment areas to create GIS layers of 1 km buffer within the catchment for each site.[28] For each buffer zone, we calculated the land use features of interest as described in our previous study[26] and presented in the Supplementary Table S4.

2.6 Statistical analysis

All statistical analyses were conducted using the R program,[44] GraphPad Prism version 9.3.1 (LaJolla, CA), and SPSS version 25.0 (Chicago, IL). To perform statistical analysis, the

concentrations of FIB and MST markers were log-transformed. The non-detects were assigned as 0 and the DNQs were assigned the value of LOQ/√2.[45] The statistical significance and variations in the physico-chemical and microbiological parameters (FIB and MST markers) across sampling sites were analyzed by Kruskal–Wallis non-parametric ANOVA using Dunn's as post-test. Cluster analysis and Pearson correlation studies were performed to determine the major driving factor responsible for the variation in concentrations of bacterial indicators of fecal pollution (FIB and MST markers) observed at Edwards aquifer region and explain the relationship between nutrient loading, fecal markers and environmental/land use variables (such as precipitation, and septic tank density). Prior to the analysis, the dataset was explored to identify redundant variables by defining the pairwise correlation among all variables using Spearman's correlations.[29] W.T, pH and DO were identified as redundant ($r<0.3$) and were not included in the analysis. The k-means agglomerative method of clustering analysis and Pearson correlation was performed as described previously.[29, 46]

3. Results and Discussion

3.1 Physico-chemical analysis of surface waters collected from Edwards aquifer

In the current study, the main physico-chemical parameters (e.g., pH, DO, NO_3-N, NO_2-N, and NH_4-N) which are known to influence or reflect the microbial activity and water pollution were studied.[47] A total of 27 sampling events were carried out for the current study and the summary (range) of physico-chemical analysis of water samples (n = 295) collected from 11 different sampling sites of the Edwards aquifer region is given in Table 1. The detailed nitrogen species concentration (NO_3-N, NO_2-N, and NH_4-N) of each water sample is shown in Supplementary Table S5. Water temperatures were in the range of 9.4 to 35.6 ^oC and were generally consistent across the sites during each sampling event. The pH values of all samples ranged from 6.4 to 9.5

and showed significant spatial and temporal variation ($p < 0.05$). Except for water samples of the L-1 site, pH levels of all the water samples were within the acceptable limits (6.5-9) as suggested by the USEPA and TCEQ for the streams and rivers.[38]. A previous study[48] indicated that a pH range of 6.5 to 8 is required to support aquatic life in natural waters and increased pH indicates possible nutrient pollution and eutrophication of water bodies. Water samples collected from the L-1 site, which is near a waste discharge outfall, frequently exceeded these pH limits (as well as USEPA recommended limits) indicating potential nutrient pollution at this location. DO is crucial to maintain biological life in the aquatic systems and could be considerably influenced by waste discharge from agriculture, industrial and municipal sewage.[49] In general, water systems with DO levels below 3 mg/L are of concern, and levels below 1 mg/L are considered hypoxic and not suitable for aquatic life.[38] In the current study, DO levels of the water samples were in the range of 2.2 to 18.2 mg/L (Table 2), and significant spatial and temporal variation ($p < 0.05$) in the DO levels was observed at the monitored sites. The DO levels at L-1 and L-4 sites of Leon Creek were intermittently below 3 mg/L, indicating potential contamination at these sites. A study conducted on the surface water quality of small streams in the Edwards Plateau of central Texas indicated that streams receiving wastewater effluents had relatively lower levels of DO and higher concentrations of nutrients.[50] Therefore, an analysis of selected nutrients in the water samples was carried out to identify nutrient pollution at monitored creeks of the Edwards aquifer.

Among the nutrients monitored, NO_3-N was frequently detected in the study area with concentrations ranging from 0 to 1.51 mg/L and exhibited significant spatial and temporal variation (Table S5.1). The median NO_3-N concentrations observed at each sampling site during the monitoring period are shown in Fig. 1 and relatively higher concentrations of NO_3-N were observed at the sites of Leon Creek with the highest at the L-2 site (0.49 mg/L). Although NO_3-N

concentrations were relatively low compared to acceptable limits suggested by USEPA (10 mg/L), the median NO_3-N concentrations at Leon, Balcones (excluding the B-1 site) and Helotes Creeks were higher than the estimated national background concentration (0.24 mg/L) for streams.[51] The sources of NO_3-N include effluents from wastewater treatment plants, leaking on-site septic systems, runoff from fertilized lawns, cropland, and animal manure storage areas.[52] As agricultural activities are relatively less in the study area,[32] the increased NO_3-N concentrations point to the contamination of these waters primarily with runoff from fertilized lawns or human and animal waste. A recent study[32] reported similar NO_3-N concentrations for the streams (including Leon and Helotes Creeks) that flow through the San Antonio segment of the Edwards aquifers region. NO_2-N is the less frequently detected nutrient monitored in the study area and the concentrations were within the acceptable limits (1 mg/L) as suggested by USEPA.[38] NO_2-N concentrations were in the range of 0 to 0.58 mg/L and showed significant spatial variation only (Fig. 1). Among the four monitored creeks, NO_2-N was more frequently detected in Leon Creek (Table S5.2). NH_4-N was detected in the water samples with concentrations ranging from 0 to 0.79 mg/L (Table S5.3) and their levels were within acceptable limits as suggested earlier.[53] However, the presence of NH_4-N in natural freshwater bodies is primarily associated with increasing anthropogenic activities, and sources like livestock manure, raw sewage, and run-off from agricultural lands are primarily responsible for their elevated concentrations.[54] The NH_4-N concentrations in the study area showed significant temporal variation and the results are consistent with the previous studies carried out at Edwards aquifer; which showed similar NH_4-N concentrations for Leon and Helotes Creeks.[55] Overall, physico-chemical analysis results indicate potential fecal contamination of waters at several sites of study area.

3.2 Performance characteristics of qPCR assays

For each qPCR assay, around 4 to 6 individual standard curves were analyzed to determine the performance characteristics of the respective assay. The range of qPCR amplification efficiency, slope, and linearity (R^2 value) for each assay is presented in Table S6 and all these parameters were within the acceptable range as per MIQE guidelines.[42] The LOD and LOQ values of the qPCR assays were in the range of 3 to 20 gene copies/reaction (Table S6). Carryover or cross-contamination was not observed in the controls used for sample processing, DNA extraction, and no template samples of qPCR assays. PCR inhibition test carried out on selected samples (12% of total samples) resulted in matching concentrations for undiluted and 10-fold diluted DNA templates, indicating PCR inhibition did not affect the amplification.

3.3 General and host-associated fecal marker trends in water samples of Edwards aquifer

From March 2019 to March 2020, a total of 295 water samples were collected from the four creeks of the Edwards aquifer region and analyzed for two FIB and eight MST fecal markers to determine the presence and source of fecal contamination. The spatial and temporal variation in the occurrence of general and host-associated fecal markers in water samples of the Edwards aquifer region is shown in Table 2 and their abundance ($\log_{10}$ gene copies/100mL) is presented in Figure 2. Among the ten fecal markers analyzed, general fecal markers (*E. coli*, *Entero1* and BacUni) were more frequently detected (> 97%) in the water samples than host-associated fecal markers. The occurrence and abundance of general and host-associated fecal markers are discussed in detail in the following subsections.

3.3.1 Prevalence and abundance of general fecal markers

Among the three general fecal markers (two FIB and one universal *Bacteroidales* MST marker), *Entero1* was more frequently detected (99 %) in the water samples with concentrations ranging from 2.53 to 5.98 $\log_{10}$ gene copies/100mL (quantifiable samples (QS), n = 260/295). The mean

concentration of *Entero1* was 3.82 ± 0.83 $\log_{10}$ gene copies/100mL and the highest concentration (Fig. 2B) was detected at the L-2 site, which is near the Dominion neighborhood and receiving waste from the neighborhood (Table S1). The concentrations of *Entero1* did not show significant statistical variation across the sampling sites ($p > 0.05$). *E. coli* was detected in 97.6 % of the water samples (Table 2) and their concentrations showed significant statistical variation ($p < 0.05$) across the sampling sites. The concentrations of *E. coli* were in the range of 2.23 to 5.85 $\log_{10}$ gene copies/100mL (QS, n = 244/295), with a mean concentration of 3.26 ± 0.79 $\log_{10}$ gene copies/100mL. The highest *E. coli* concentration (Fig. 2A) was detected at the B-1 site, which is located in a rural area on the northern border of Bexar County. The universal *Bacteroidales* marker, BacUni, was detected in 98.3% of water samples with concentrations ranging from 2.85 to 6.81 $\log_{10}$ gene copies/100mL (QS, n = 280/295). The mean abundance of BacUni was 4.70 + 0.81 $\log_{10}$ gene copies/100mL and their highest concentrations (Fig. 2C) were frequently detected at the L-4 site (located near an outfall from a student dormitory close to the University of Texas at San Antonio). The BacUni marker concentrations showed significant variation ($p < 0.05$) among the sampling sites.

Overall, the high detection frequency of general fecal markers indicates the presence of fecal contamination at the monitored creeks of Edwards aquifer; although *E. coli* and *Enterococcus* have been reported to survive and grow outside human and animal guts, such as soil and aquatic environments.[56] However, the presence of universal *Bacteroidales* markers, BacUni, confirms recent fecal contamination at the monitored sites. Furthermore, the frequent detection of general fecal markers with high concentrations at several sites following storm events indicates runoff can introduce and elevate fecal contamination; for instance, significant rainfall events (>2 inches precipitation, Table S3) occurred before the sampling events dated on 5/10/2019 and 10/25/2019

and high concentrations of general fecal markers were detected in most of the samples collected from these sampling events. Several previous studies indicated similar elevated concentrations of FIB and MST markers in environmental waters following storm events.[57, 58] But, it was reported that storm events can introduce untreated sewage and non-point fecal sources entry from different animals such as dogs, birds, and cattle, which can significantly increase the occurrence of pathogenic microorganisms.[43, 59] Therefore, as general fecal markers do not specify the source of fecal contamination, accurate identification of fecal sources and hotspots of fecal contamination are necessary to identify potential public health risks and implement BMPs at the Edwards aquifer.

3.3.2 Prevalence and abundance of human-associated MST markers

The human-associated MST markers (BacHum and HF183) were less frequently detected in the water samples of the study area (Table 2). BacHum was detected in 20.7 % of water samples with concentrations ranging from 2.22 to 2.89 $\log_{10}$ gene copies/100mL (QS, n = 12/295), while HF183 was detected in 15.3% of samples with concentrations ranged from 2.22 to 3.1 $\log_{10}$ gene copies/100mL (QS, n = 20/295). The mean concentrations of BacHum and HF183 were 2.56 ± 0.20 and 2.43 ± 0.21 $\log_{10}$ gene copies/100mL and their highest concentrations (Fig. 2D & 3E) were detected at sites B-4 and L-3, respectively. BacHum concentrations were not statistically significant, while HF183 showed significant variation in concentrations across the sites ($p < 0.05$). The human-associated MST markers were frequently detected at a quantifiable range in L-3, S-2, and H-1 sites; although, they were also quantified sporadically at B-2 and B-4 sites. The human population in the San Antonio segment of the Edwards aquifer is rapidly growing, primarily in the communities at the northern side such as Helotes, Fair Oaks Ranch, Boerne, Timber wood Park, and Scenic Oaks. This population growth requires an increased number of wastewater treatment plants to treat the sewage. But it was reported that a significant amount of the population in this

region uses onsite sewage facilities (OSSFs) to treat the wastewater; for instance, around 45% of Fair Oaks Ranch residential properties use OSSFs.[60] It has been reported[32] that land application of treated wastewater and septic systems in the San Antonio segment of the Edwards aquifer is responsible for increased NO_3-N levels in the streams. In the current study, the frequent detection of human fecal markers at the L-3 site could be related to the human fecal source or waste entry from the Dominion neighborhood that is located close to the site; while their detection in the remaining sites (S-2, H-1, B-2, and B-4) can be attributed to the septic leakage or land application of treated wastewater. Among these sampling sites, the presence of human fecal contamination at B-4 site is a significant public health concern as this site is located in recharge zone of Edwards aquifer region.

3.3.3 Prevalence and abundance of ruminant and cattle-associated MST markers

The ruminant and cattle-associated MST markers (Rum2Bac and BacCow) were the second most frequently detected host-associated MST markers in the study area (Table 2). Rum2Bac marker was detected in 63.4 % of water samples with concentrations ranging from 2.22 to 3.6 $\log_{10}$ gene copies/100mL (QS, n = 47/295). The mean concentration of Rum2Bac markers detected in the water samples was 2.49 ± 0.53 $\log_{10}$ gene copies/100mL and their highest concentration (Fig. 2F) was detected at the B-2 site, which is located in a rural area in the northern boundary of Bexar County (Table S1). Similarly, the BacCow marker was detected in 63.7 % of water samples collected from the Edwards aquifer region. The concentration of BacCow markers ranged from 2.22 to 5.2 $\log_{10}$ gene copies/100mL (QS, n = 85/295), with a mean of 2.87 ± 0.57 $\log_{10}$ gene copies/100mL. The highest concentration (Figure 2G) of BacCow markers was detected at the B-4 site, which is in the recharge zone of Edwards aquifer and located near the City of Fair Oaks Ranch in the northern boundary of Bexar County. BacCow marker concentrations showed

significant statistical variation ($p < 0.05$) across sampling sites, while statistical significance was not observed for Rum2Bac marker concentrations.

During sampling events, Rum2Bac and BacCow markers were detected at most of the sites following storm events (dated 5/10/2019 and 10/25/2019), indicating stormwater runoff can significantly introduce cattle and ruminant fecal sources into the streams of the Edwards aquifer region. As mentioned earlier, numerous studies have documented the non-point source entry of fecal sources into the environmental waters during storm events.[61, 62] The contributing zone of the Edwards Aquifer region has a large number of ranches with cattle population, and 5.8 to 9.6 % of land in the communities on the northern side of San Antonio (such as Helotes and Fair Oaks Ranch) is used for pasture/hay purpose.[32] Furthermore, Bexar county is the natural habitat for several ruminant wildlife animals such as deer and elk.[26] Therefore, the feces from these animal sources can significantly influence the microbial water quality during storm events. The frequent detection of BacCow marker at a quantifiable range in most of the sampling sites collected from Balcones, Helotes and San Geronimo Creeks accords with the land use pattern. However, their detection at Leon Creek sites, where cattle population is very little or none, could be due to the cross-reactivity of BacCow marker with ruminant wildlife or dog feces. The authors[16] who developed this assay reported the cross reactivity of BacCow markers with horse fecal samples. Furthermore, previous studies also reported the cross-reactivity of this marker with other hosts such as dog, deer and pig fecal sources.[63, 64]

3.3.3 Prevalence and abundance of canine-associated MST markers

Canine-associated MST marker (BacCan) was detected in 34.9 % of water samples collected from the Edwards aquifer region (Table 2). The concentrations of BacCan markers were in the range of 2.22 to 4.5 $\log_{10}$ gene copies/100mL (QS, n = 43/295) with a mean of 3.04 ± 0.63 $\log_{10}$ gene

copies/100mL. Significant statistical variation ($p < 0.05$) in the concentrations of BacCan markers was observed among the sampling sites and the highest concentration (Fig. 2H) was detected at the B-2 site. The BacCan markers were frequently detected at quantifiable range in the L-2, L-3 and B-4 sites and they were also detected intermittently at quantifiable range in L-1, L-4, B-1, B-2, B-3, and S-2 sites. Among these, L-1, B-3 and B-4 sites are located in the recharge zone of Edwards aquifer and canine fecal contamination of these sites indicates potential human health risk. Similar to Rum2Bac and BacCow markers, the BacCan marker was also detected in most of the samples collected after storm events (dated 5/10/2019 and 10/25/2019); thus, further confirming that stormwater runoff can contribute to the non-point source of fecal entry into the streams of the Edwards aquifer region. According to National Pet Owners Survey, about 57 % of households in San Antonio city have pets (of which more than 50 % are dogs) and approximately 34,363 unrestrained dogs exist at any given time in San Antonio City.[65] Therefore, dog feces could significantly contribute as a non-point source of fecal contamination during storm events. Comparatively, BacCan markers were more frequently detected at the sampling sites of Leon Creek than at the other sites (Fig. 2H). A 20 miles multi-use trail that is adjacent to Leon Creek allows dog walking activities[66] and the result from the current study emphasizes the poor pet waste management in this area.

3.3.4 Prevalence and abundance of avian-associated MST markers

Among the avian-associated MST markers, GFD was more frequently detected (90.5 %) in water samples of the Edwards aquifer region than the Chicken/Duck-Bac marker (23.4 %). The low detection frequency of Chicken/Duck-Bac markers in water samples was anticipated as these markers are designed for detecting *Bacteroidales* in chicken and duck fecal sources and previous studies indicated that *Bacteroidales* are less frequent in avian gut or feces.[67, 68] The high detection

frequency of the GFD marker is possible as these markers detect *Helicobacter sp*. that is present in a wide range of avian species including seagull, chicken, duck, and waterfowls.[24] GFD marker was the most frequently detected host-associated MST marker in the study area with concentrations ranging from 2.22 - 6.6 $\log_{10}$ gene copies/100mL (QS, n = 197/295) and a mean of 3.03 ± 0.71 $\log_{10}$ copies/100mL. Chicken/Duck-Bac markers were detected in the range of 2.22 to 4.9 $\log_{10}$ gene copies/100ml (QS, n = 25/295), with a mean of 2.93 ± 0.66 $\log_{10}$ gene copies/100mL. The highest concentration of both markers (Fig. 2I & 2J) was detected at the L-2 site and significant statistical variation ($p < 0.05$) in concentration across the sampling sites was observed for both markers. According to Texas Parks and Wildlife, Texas has recorded more species of birds (over 615 species) than any other state in the US and 50 % of these are migratory birds that passage through Bexar County during the spring and fall/winter seasons.[69] These migratory birds move to the northern hemisphere in the spring and to the south during the fall or winter seasons, during which their passage through Texas takes place. While spring migration/passage is shorter (around four weeks) starting around mid-April to mid-May, the fall migration/passage spans a longer time range that starts from late August to mid-November.[70] The higher frequency of GFD marker detection in the study area during fall is consistent with the passage pattern of these migratory birds. Although occurrence of pathogens was reported in bird feces, exposure to avian fecal sources is considered relatively less harmful to humans than exposure to other sources of fecal sources, especially humans.[27, 71]

In summary, our results indicate that GFD, Rum2Bac, BacCow, and BacCan markers were the most frequently detected host-associated markers in this study, suggesting higher animal fecal contamination in the Edwards aquifer region. Similar to previous studies, our results also reveal that stormwater runoff could significantly transport animal feces to the receiving waters.[62]

Globally, animal feces contribute to a larger amount of fecal material than human fecal waste, and animal feces exposure has been recognized as the main route of contamination in the environment.[72] Animal feces can act as a zoonotic pathogens source and studies have shown that cattle, dog and avian feces contain a broad range of zoonotic pathogens such as *E. coli* O157:H7, *Campylobacter jejuni*, and *Salmonella* spp.[73-75] Therefore, based on the results, we can conclude the potential presence of zoonotic pathogens risk in the waters of the study area.

3.4 Relationship between nutrients, FIB, MST, and environmental/land use factors

Several previous studies carried out on understanding the relationship between fecal contaminants and nutrient loadings reported a masked or less correlation[76] and suggested that considering the similarities and differences between sampling sites and combining environmental/land use factors in the analysis could provide a better understanding of their relationship and potential fecal sources.[29, 46] In the current study, when nutrients, FIB, and MST markers data from all sampling sites were analyzed, a similar (less or masked) correlation was observed between these groups (Supplementary Table S7). NO_3-N showed a significant positive correlation with BacCan (Rho (r) = 0.67, p=0.02) and CDBac (r=0.80, p=0.003); NH_4-N was positively correlated with BacCow (r=0.60, p=0.008); E. coli correlated with BacUni (r=0.88, p=0.00). In this regard, spatial clustering of sampling sites was performed using four different data categories (FIB markers, MST markers, precipitation, and septic tank density), and the correlation between nutrients and fecal markers in clustered sampling sites was examined.

When cluster analysis was performed using the FIB (*E. coli* and *Entero1*) markers, only two clusters were generated (Table 3) with FIB markers concentrations at 0-5.63 and 0-5.98 Log_{10} gene copies/100mL in cluster-1 and cluster-2, respectively. The correlation analysis performed on nutrients, FIB and MST markers data of cluster-1 sampling sites (B-2 and B-3) indicated that NO_3-

N showed a significant positive correlation with BacUni (r=0.91, p=0.001), BacCan (r = 0.66, p=0.04) and CDBac (r=0.83, p=0.005); *E. coli* showed correlation with BacUni (r=0.67, p=0.04) only; *Entero1* was positively correlated with BacCow (r=0.70, p=0.03) only. For cluster-2 (consisting of remaining 9 sites, Table 3), NO_2-N showed negative correlation with *E. coli* (r= -0.83, p=0.019) and NO_3-N was positively correlated with Rum2Bac (r=0.76, p=0.04); NH_4-N showed significant positive correlation with GFD (r=0.769, p=0.04) and *Entero1* was negatively correlated with GFD (r=-0.756, p=0.04). Although the correlation was improved (particularly for sampling sites of cluster-1), the results could not provide a better relationship among nutrients, FIB, and MST markers in these clusters. For instance, NO_3-N was significantly correlated with BacCan and CDBac only, indicating these fecal sources could be the source of NO_3-N at these sites;[54] However, the more abundant MST markers (such as Rum2Bac and BacCow) showed no correlation with nutrients at these sites. Therefore, cluster analysis of sampling sites based on FIB, which occurs in human, animal, avian, and natural environments,[77-79] may not provide accurate spatial clustering and appropriate relationship among nutrients and fecal markers.

Spatial clustering performed with MST markers generated three clusters (Table 3) and the concentration of MST markers for cluster-1, 2, and 3 sites are 0-6.06, 0-6.65, and 0-6.82 $\log_{10}$ gene copies/100mL, respectively. The correlation analysis results for cluster-1 sites (B1, B-3, S-1) indicated NO_2-N was negatively correlation with GFD (r=-0.96, p=0.03), while NO_3-N showed positive correlation with *Entero1* (r=0.44, p=0.04), Rum2Bac (r=0.50, p=0.02), and BacCan (r=0.60, p=0.004); NH_4-N was positively correlated with CDBac (r=0.70, p=0.001); *E.coli* correlated with *Entero1* (r=0.22, p=0.04), BacUni (r=0.46, p=0.00), BacCow (r=0.50, p=0.00), BacCan (r=0.22, p=0.04), and GFD (r=0.43, p=0.00); *Entero1* showed positive correlation for BacUni (r=0.68, p=0.00) and GFD (r=0.358, p=0.00). For cluster-2 sites (L-1, B-2 B-4), only NO_2-

N was positively correlated with GFD (r=0.97, p=0.005) and in case of cluster-3 sites, NO_3-N showed correlation with BacUni (r=0.94, p=0.01) and *Entero1* was positively correlated with BacCow (r=0.96, p=0.00). Overall, the correlation of NO_3-N, *E. coli*, and *Entero1* with more abundant MST markers (Rum2Bac, BacCow, BacCan, GFD) of cluster-1 sampling sites provide a better understanding of the relationship between nutrients, FIB, and MST markers at these sites.[58, 80] But, the sampling sites of cluster-2 and 3 showed less correlation, indicating MST marker-based spatial clustering may not explain the relationship among nutrients and fecal markers appropriately.

Cluster analysis carried out with precipitation data resulted in three clusters (Table 3), which can be classified as low (0-1in), medium (0-3.1 in), and high (0-6.9 in) precipitation clusters. The correlation analysis results of these clusters showed a better relationship between nutrients, FIB, and MST markers (Supplementary Table S8.1). For cluster-1 sampling sites (L-1 and H-1) which received less amount of precipitation, NO_3-N showed a positive correlation with BacHum (r=0.37, p=0.04) and Rum2Bac (r=0.44, p=0.01), indicating human and ruminant fecal contamination contributed to the NO_3-N at these locations.[81] These results are convincing as significant ruminant and human fecal contamination was observed at L-1 and H-1 sites (Table 2). *Entero1* was positively correlated with Rum2Bac but negatively correlated with NO_2-N. Additionally, *E. coli* showed a positive correlation with 6 MST markers (BacUni, HF183, BacCow, BacCan, CDBac, and GFD; r and p values were given in Supplementary Table S8.1) indicating human, cow, dog, and avian fecal sources are the contributor of *E. coli* at these locations.[82] Similarly, for cluster-2 sampling sites (S-1, S-2), NO_3-N was positively correlated with *Entero1*, BacCan, and GFD; While *E. coli* was correlated with BacUni, BacCow, and GFD, *Entero1* correlated with BacUni, Rum2Bac, and BacCow. For sampling sites (L-2, L-3, L-4, B-1,

B-2, B-3, and B-4) of cluster-3 that received high precipitation, more correlation between nutrients (NO_3-N and NH_4-N), FIB (*E. coli*) and MST markers (BacUni, HF183, BacHum, Rum2Bac, BacCow, CDBac) was observed (Supplementary Table S8.1). These results highlight that the multiple fecal sources are responsible for the increase of nutrients and FIB during rain events, which is consistent with previous studies.[83]

Spatial clustering carried out with septic tank density data generated three clusters (Table 3) representing cluster-1, 2, and 3 at 1-10 (low density), 15-30 (medium density), and 36-51 (high density) septic tank units/km^2, respectively. The clustered sampling sites in this category showed the highest correlation between nutrients, FIB, and MST markers (Supplementary Table S8.2). For cluster-1 (L-4, B-1, B-2, S-1, S- 2) sampling sites that primarily displayed higher concentrations of ruminant, cow, and avian markers (Table 2), NO_3-N was positively correlated with Rum2Bac, BacCan, and GFD; NH_4-N showed correlation with E. coli; While *E. coli* was positively correlated with 6 MST markers (BacUni, HF183, BacCow, BacCan, CDBac, and GFD), *Entero1* showed a correlation with four MST markers (BacUni, BacHum, Rum2Bac, and BacCow). In case of cluster-2 sites (L-1, L-2, L-3), NO_3-N was positively correlated with *Entero1*, BacHum, and Rum2Bac; While NH_4-N showed positive correlation with BacUni and GFD, NO_2-N was negatively correlated with *Entero1*; Except for Rum2Bac, *E. coli* showed a significant positive correlation with all the MST markers tested (Supplementary Table S8.2) and *Entero1* was correlated with 5 MST markers (BacUni, BacHum, HF183, Rum2Bac, BacCan). For cluster-3 sites (B-3 B-4 H-1), NO_3-N showed positive correlation with *Entero1* and Rum2Bac, and negative correlation with NH_4-N and CDBac; While *E. coli* was correlated with 6 MST markers (BacUni, HF183, Rum2Bac, BacCow, BacCan, and GFD), *Entero1* correlated with 4 MST markers (BacUni, BacHum, Rum2Bac, and GFD). Among cluster-3 sites, H-1 site has the highest septic

tank density and highest human fecal markers (Table 2) detection was observed at this site. Overall, the high correlation between nutrients and fecal markers in sampling sites of these clusters indicates the efficiency of septic tank-based clustering. A recent study[29] also reported similar findings and indicated that septic tank density/land use could help in prediction of fecal pollution.

In summary, spatial clustering based on precipitation and septic tank density provided a better correlation among nutrients, FIB, and MST makers, indicating an improved understanding of the relationship between nutrient loading and fecal contamination can be achieved when similarities and differences between sampling sites and environmental factors are incorporated in the study.[29] However, the significance of spatial clustering is that it can help in prioritizing the sampling sites for fecal contamination monitoring, providing cost-effective solutions. For instance, the S-1 and S-2 sites of San Geronimo Creek and, the B-1 and B-2 sites of Balcones Creek (which are closer to each other, Fig.1) showed similar nutrients and fecal markers results and were clustered in the same cluster when analyzed based on precipitation and septic tank density, indicating only one site from each of these Creeks is sufficient for monitoring fecal pollution in future studies.

3.5 Implications of water quality on the Edwards Aquifer

Several studies conducted on the physico-chemical characteristics of waters from recharge and contributing zones of Edwards aquifer indicated potential sewage and animal waste entry into creeks and rivers, emphasizing possible fecal contamination of the water bodies.[3, 32] However, studies on identifying fecal pollution and its sources in the Edwards Aquifer region are rare and, to our knowledge, none in Helotes and San Geronimo Creeks.[26] In this regard, the current study was carried out to identify the sources of fecal pollution at Leon, Balcones, San Geronimo, and

Helotes Creeks that flow through recharge and contributing zones of the Edwards aquifer region by applying MST approach together with physico-chemical analysis.

The physico-chemical analysis of water indicated NO_3-N levels were higher than the estimated national background concentration at several sites and the presence of NH_4-N pointed to the potential fecal contamination of sampling sites monitored in the Edwards aquifer region. Detection of FIB and BacUni markers in more than 97% of monitored water samples confirm the presence of fecal contamination in the Edwards aquifer region. Among the host-associated fecal markers, human-associated fecal markers (BacHum and HF183) were less frequently detected in the study area. However, the occurrence of human fecal markers at the L-3, B-4, and H-1 sites could be related to the entry of human feces or septic leakage as high septic tanks density (Supplementary Table S4) was observed at these sites and human fecal markers detection at B-2 and S-2 sites could be related to the entry of treated wastewater applied to the lands. A study reported that around 3 million liters of wastewater spillage occurred in the San Antonio segment recharge zone of the Edwards aquifer during 2004 to 2012.[84] Results highlight the need for the continuous monitoring of human fecal markers and human fecal-associated pathogens in the surface and ground waters sources at these sites or discourage the permits for septic systems and land application of treated wastewater in the San Antonio segment's recharge zone of Edwards aquifer. The frequent detection of ruminant and cattle-associated MST markers in the Balcones, Helotes, and San Geronimo Creeks sites suggests proper control and management efforts related to livestock ranches are required. Canine-associated MST markers were primarily detected in the Leon Creek sites, pointing to the poor pet waste management practices in the area. GFD markers were the most abundant host-associated MST markers detected in the study area and results indicate significant avian fecal pollution. However, as Bexar County is the natural habitat for

several resident and migratory birds, future studies on the occurrence of avian-associated pathogens in the study area are necessary to identify the human health risks and further studies to understand the decay rates of the GFD marker in environmental water samples are required for proper identification of public health risks.

There are limited TCEQ water quality regulations for the contributing zone of the Edwards aquifer region compared to the recharge zone; this is with a premise that water from the contributing zone does not recharge the Edwards aquifer directly and the contributing zone's role is merely to transport surface water to the recharge zone of Edwards aquifer where it enters the subsurface. However, it was reported that the Edwards aquifer is significantly recharged by water infiltrating the contributing zone because of higher hydraulic communications, and in many areas, the distinction between recharge and contributing zones of the Edwards aquifer is not clear.[85] In the current study, water samples collected from contributing zones (primarily from sites that are within 2 miles away from the recharge zone) in Leon, Balcones, Helotes, and San Geronimo Creeks showed significant fecal contamination, suggesting public health risks.

Therefore, as the Edwards aquifer is a karst-dominated terrain and the presence of human and animal fecal contamination in the creeks of recharge and contributing zones is confirmed, further studies evaluating the microbial quality of groundwater sources at these sampling sites are crucial to determine human health risk, more importantly during storm events.

4. Conclusions

We systematically analyzed waters collected from four karst-dominated creeks of the Edwards aquifer for a range of physico-chemical and microbiological (FIB and MST) parameters and identified the relationship of fecal markers with nutrients in different environmental/land-use conditions. The main conclusions of this study are summarized as follows: 1) though monitored

nitrogen species (NO_3-N, NO_2-N, and NH_4-N) are within the acceptable range, their elevated concentrations point to the potential nutrient pollution and possible fecal contamination. 2) avian, ruminant, and dog fecal sources are the primary sources of fecal pollution at monitored sites of the Edwards aquifer region and their presence at recharge sites indicate signifcant public health concern 3) spatial clustering of sampling sites suggested that temporal and spatial variation in the nutrients and fecal markers could be primarily related to precipitation and septic tank density respectively and clustered sampling sites based on precipitation and septic tank density categories showed a better correlation among nutrients, FIB, and MST markers. Furthermore, spatial clustering results indicated that it can help in prioritizing the sampling sites for fecal monitoring, providing cost-effective solutions.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 1. Summary of Physico-chemical analysis (range- minimum to maximum) of water samples collected from eleven different sites of the study area.

Parameter	Sampling Site											*p* value	
	L-1	**L-2**	**L-3**	**L-4**	**B-1**	**B-2**	**B-3**	**B-4**	**S-1**	**S-2**	**H-1**	**Spatial**	**Temporal**
W. T (^oC)	12.2-29.1	14.5-31.8	12.9-29.5	10.5-28.6	11.1-30.2	9.4-29.1	12.9-32.1	13.5-35.6	12.3-29.6	9.8-28.2	9.6-27.0	0.3350[a]	<0.0001[b]
pH	6.4-9.5	6.8-8.1	6.5-8.1	6.9-8.1	6.7-8.1	6.8-8.6	6.7-8.3	6.8-8.5	6.7-8.4	6.6-8.1	6.6-8.0	<0.0001[b]	<0.0001[b]
DO (mg/L)	2.2-12.4	5.1-18.2	3.7-12.9	2.7-12.6	5.8-11.9	4.1-13.4	6.4-12.4	5.6-17.9	7.9-10.6	6.8-11.9	5.1-9.8	<0.0001[b]	<0.0001[b]
NO_3-N (mg/L)	0-1.48	0-1.30	0-1.51	0-1.18	0-0.29	0-1.09	0-0.88	0-1.42	0-0.08	0-0.42	0-0.73	0.0051[b]	<0.0001[b]
NO_2-N (mg/L)	0-0.42	0-0.12	0-0.12	0-0.58	0-0.02	0-0.05	0-0.03	0-0.09	0	0-0.02	0-0.01	0.0003[b]	0.1305[a]
NH_4-N (mg/L)	0-0.04	0-0.22	0-0.05	0-0.15	0-0.04	0-0.17	0-0.11	0-0.79	0-0.05	0-0.20	0-0.15	0.4336[a]	0.0462[b]

[a] statistically not significant; [b] statistically significant at $p \leq 0.05$.

Table 2. Detection frequency of general and host-associated fecal markers in the water samples collected from different sites of the study area.

Sampling site	No. of samples tested	No. of positive samples (%) [a]/ No. of quantifiable samples									
		E. coli	Entero1	BacUni	BacHum	HF183	Rum2Bac	BacCow	BacCan	Chicken/ Duck-Bac	GFD
L-1	27	26 (96.3)/22	27 (100)/21	27 (100)/25	3 (11.1)/2	4 (14.8)/2	18 (66.7)/6	15 (55.6)/7	11 (40.7)/4	1 (3.7)/0	24 (88.9)/17
L-2	27	27 (100)/26	27 (100)/26	27 (100)/26	5 (18.5)/1	3 (11.1)/0	19 (70.4)/2	19 (70.4)/11	14 (51.9)/9	16 (59.3)/6	25 (92.6)/15
L-3	27	27 (100)/23	27 (100)/26	27 (100)/27	8 (29.6)/0	9 (33.3)/7	17 (63.0)/3	19 (70.4)/9	14 (51.9)/9	14 (51.9)/9	26 (96.3)/20
L-4	27	27 (100)/24	27 (100)/25	27 (100)/25	3 (11.1)/0	1 (3.7)/0	16 (59.3)/5	13 (48.1)/5	8 (29.6)/3	1 (3.7)/0	25 (92.6)/16
B-1	27	27 (100)/19	26 (96.3)/25	27 (100)/27	2 (7.4)/0	1 (3.7)/0	18 (66.7)/2	12 (44.4)/2	7 (25.9)/3	2 (7.4)/0	25 (92.6)/20
B-2	26	26 (100)/21	26 (100)/23	26 (100)/25	7 (26.9)/3	3 (11.5)/1	16 (61.5)/5	13 (50.0)/6	8 (30.8)/2	3 (11.5)/0	25 (96.2)/21
B-3	26	22 (84.6)/16	24 (92.3)/19	21 (80.8)/19	3 (11.5)/1	1 (3.8)/0	14 (53.8)/5	10 (38.5)/5	8 (30.8)/3	7 (26.9)/2	18 (73.1)/9
B-4	27	27 (100)/26	27 (100)/25	27 (100)/27	5 (18.5)/2	5 (18.5)/2	18 (66.7)/5	22 (81.5)/13	11 (40.7)/6	9 (33.3)/7	26 (96.3)/21
S-1	27	27 (100)/22	27 (100)/25	27 (100)/26	6 (22.2)/0	2 (7.4)/0	16 (59.3)/6	21 (77.8)/6	2 (7.4)/0	2 (7.4)/0	23 (85.2)/17
S-2	27	27 (100)/23	27 (100)/22	27 (100)/27	5 (18.5)/1	6 (22.2)/4	18 (66.7)/3	23 (85.2)/11	10 (37.0)/3	8 (29.6)/0	24 (88.9)/18
H-1	27	25 (92.6)/22	27 (100)/23	27 (100)/26	14 (51.9)/2	10 (37.0)/4	17 (63.0)/5	21 (77.8)/10	10 (37.0)/1	6 (22.2)/1	25 (92.6)/23
Total	295	288 (97.6)/244	292 (99.0)/260	290 (98.3)/280	61 (20.7)/12	45 (15.3)/20	187 (63.4)/47	188 (63.7)/85	103 (34.9)/43	69 (23.4)/25	267 (90.5)/197

[a] considering DNQs as positive samples.

Table 3. Cluster analysis results for fecal & MST markers, precipitation, and septic tank density data. Sampling sites were clustered into groups (up to three) and can be characterized as low, moderate, and high relative value categories.

Parameter	Cluster 1 (Min-Max)[a]	Cluster 2 (Min-Max)	Cluster 3 (Min-Max)
Fecal Markers	B-2, B-3 (0 – 5.63 Log_{10} copies/ 100mL)	L-1, L-2, L-3, L-4, B-1, B-4, S-1, S-2, H-1 (0 - 5.98 Log_{10} copies/100mL)	
MST Markers	B-1, B-3, S-1 (0 - 6.06 Log_{10} copies/ 100mL)	L-1, B-2, B-4 (0 – 6.65 Log_{10} copies/ 100mL)	L-2, L-3, L-4, S-2, H-1 (0 - 6.81 Log_{10} copies/ 100mL)
Precipitation	L-1, H-1 (0 - 1 inch)	S-1, S-2 (0 - 3.1 inches)	L-2, L-3, L-4, B-1, B-2, B-3, B-4 (0- 6.9 inches)
Septic Tank Density	L-4, B-1, B-2, S-1, S-2 (1-10 units/km^2)	L-1, L-2, L-3 (15-30 units/km^2)	B-3, B-4, H-1 (36-51 units/km^2)

[a] minimum to maximum concentrations/values at these locations.

Figure Legends

Figure 1. Map showing sampling sites selected from four creeks located in the Edwards Aquifer recharge and contributing zones in Bexar County, Texas, USA.

Figure 2. Heat map showing the spatial and temporal variation of *E. coli* (A), *Entero1* (B), BacUni (C), BacHum (D), HF183 (E), Rum2Bac (F), BacCow (G), BacCan (H), Chicken/Duck-Bac (I) and GFD (J) markers with concentrations (Log_{10} copies per 100 mL) above LOQ in the water samples collected from different sites of Leon (L), Balcones (B), San Geronimo (S), and Helotes (H) creeks. "X" indicates sample was not tested.

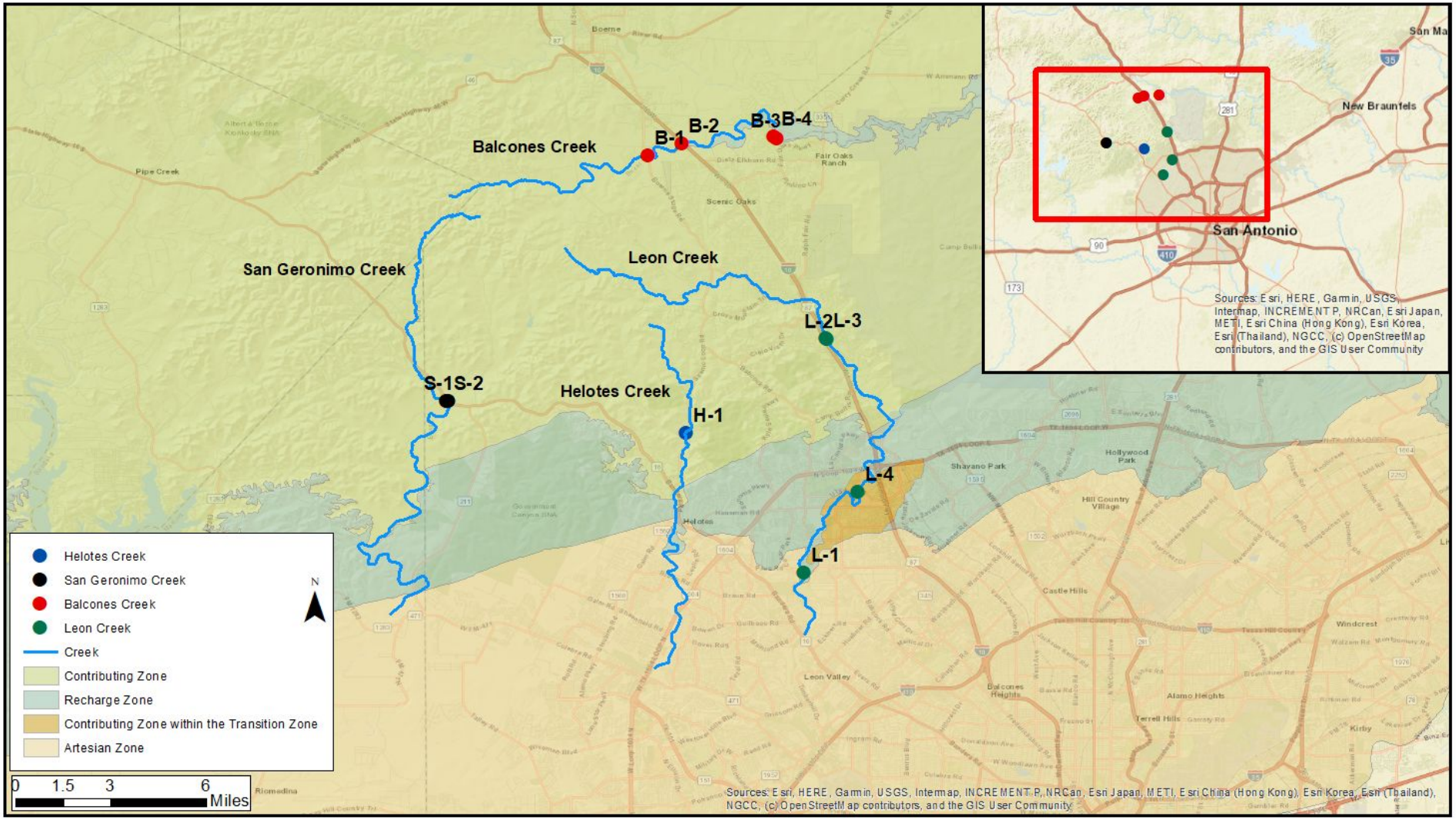
Balcones Creek
B-1
B-2
B-3
B-4
San Geronimo Creek
Leon Creek
L-2
L-3
S-1
S-2
Helotes Creek
H-1
L-4
L-1
San Antonio
New Braunfels
Helotes Creek
San Geronimo Creek
Balcones Creek
Leon Creek
Creek
Contributing Zone
Recharge Zone
Contributing Zone within the Transition Zone
Artesian Zone
N
0 1.5 3 6 Miles
Sources: Esri, HERE, Garmin, USGS, Intermap, INCREMENT P, NRCan, Esri Japan, METI, Esri China (Hong Kong), Esri Korea, Esri (Thailand), NGCC, (c) OpenStreetMap contributors, and the GIS User Community

Figure 1

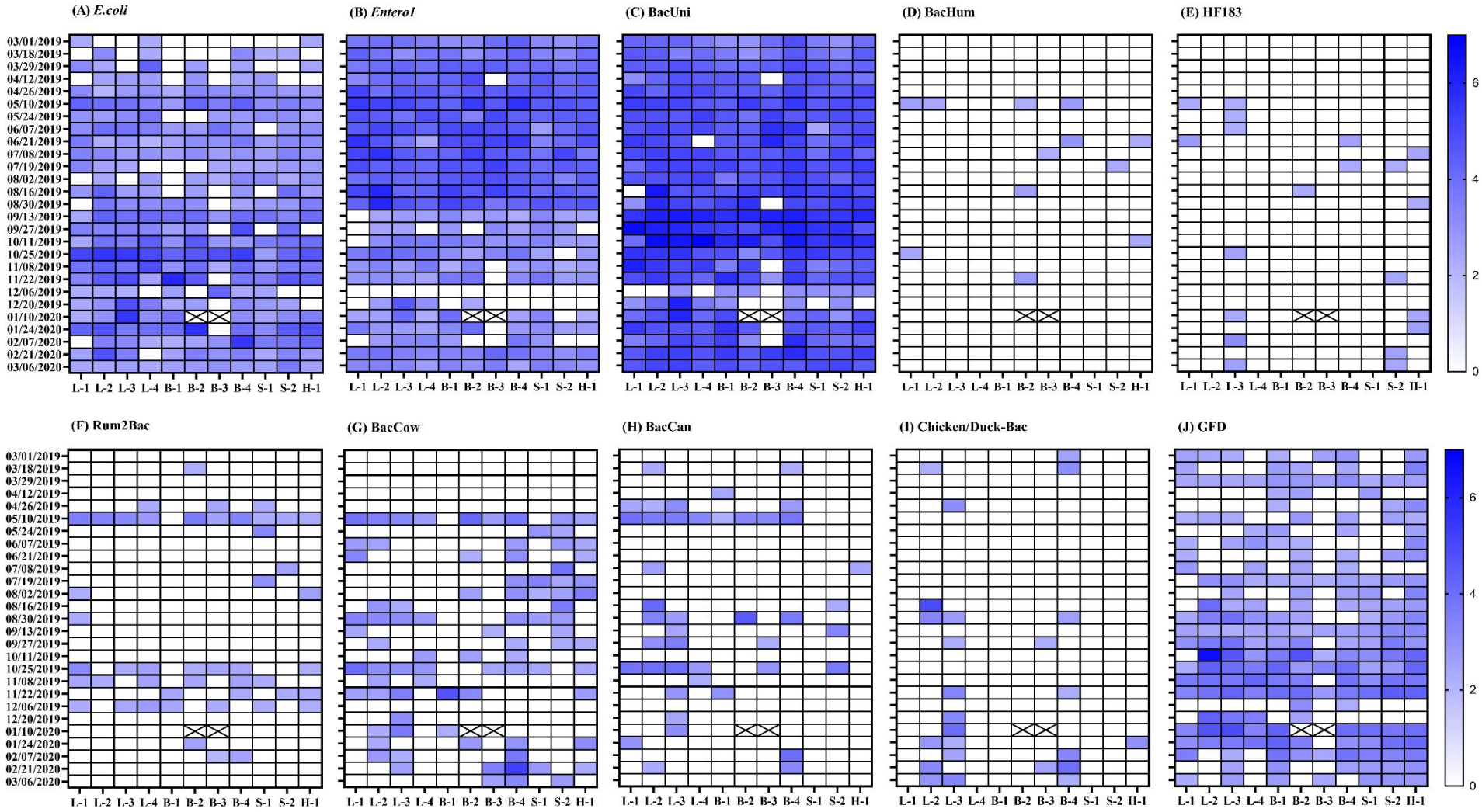
(A) E.coli
(B) Entero1
(C) BacUni
(D) BacHum
(E) HF183
(F) Rum2Bac
(G) BacCow
(H) BacCan
(I) Chicken/Duck-Bac
(J) GFD
03/01/2019
03/18/2019
03/29/2019
04/12/2019
04/26/2019
05/10/2019
05/24/2019
06/07/2019
06/21/2019
07/08/2019
07/19/2019
08/02/2019
08/16/2019
08/30/2019
09/13/2019
09/27/2019
10/11/2019
10/25/2019
11/08/2019
11/22/2019
12/06/2019
12/20/2019
01/10/2020
01/24/2020
02/07/2020
02/21/2020
03/06/2020
L-1 L-2 L-3 L-4 B-1 B-2 B-3 B-4 S-1 S-2 H-1
0
2
4
6

Figure 2