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Residential urban stormwater runoff: A comprehensive profile of microbiome and antibiotic resistance

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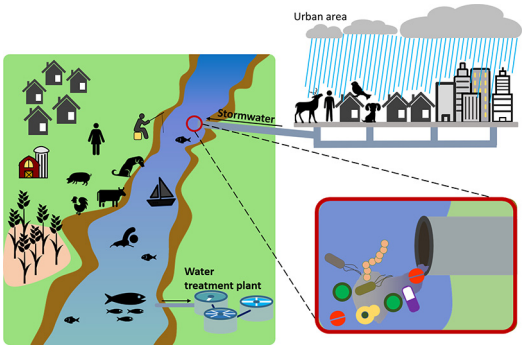
HIGHLIGHTS

- Extreme precipitation contributed to high concentrations of *E. coli* in stormwater.
- Stormwater-derived microbiome and resistome was profiled with metagenomics.
- Among antibiotic resistance (AR) genes, β-lactam resistance was ubiquitously detected.
- Ruminant (deer)- and human-associate fecal bacteria contamination was dominant.
- Stormwater can contribute to pathogen and AR transmission in nearby surface water.

GRAPHICAL ABSTRACT



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ABSTRACT

Non-point stormwater runoff is a major contamination source of receiving waterbodies. Heightened incidence of waterborne disease outbreaks related to recreational use and source water contamination is associated with extreme rainfall events. Such extreme events are predicted to increase in some regions due to climate change. Consequently, municipal separate storm sewer systems (MS4s) conveying pathogens to receiving waters are a growing public health concern. In addition, the spread of antibiotic resistance genes (ARGs) and antibiotic resistant bacteria in various environmental matrices, including urban runoff, is an emerging threat. The resistome and microbiota profile of MS4 discharges has yet to be fully characterized. To address this knowledge gap, we first analyzed the relationship between rainfall depth and intensity and *E. coli* densities (fecal indicator) in stormwater from four MS4 outflows in Columbus, Ohio, USA during the spring and summer of 2017. Microbial source tracking (MST) was conducted to examine major fecal contamination sources in the study sewersheds. A subset of samples was analyzed for microbial and resistome profiles using a metagenomic approach. The results showed a significant positive relationship between outflow *E. coli* density and rainfall intensity. MST results indicate prevalent fecal contamination from ruminant populations in the study sites (91% positive among the samples tested). *Protobacteria* and *Actinobacteria* were two dominant bacteria at a phylum level. A diverse array of ARGs and potentially pathogenic bacteria (e.g. *Salmonella enterica* Typhimurium), fungi (e.g. *Scedosporium apiospermum*), and protists (e.g. *Acanthamoeba palestinensis*) were found in urban stormwater outflows that discharge into adjacent

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streams. The most prevalent ARGs among samples were β-lactam resistance genes and the most predominant virulence genes within bacterial community were related with *Staphylococcus aureus*. A comprehensive contamination profile indicates a need for sustainable strategies to manage urban stormwater runoff amid increasingly intense rainfall events to protect public and environmental health.

1. Introduction

Urbanization has caused critical changes in local landscape, land use, air quality, and hydrological cycle (McGrane, 2016). Previous studies showed that modifications of land use related with urbanization have increased stormwater runoff volumes and peak flows (Barbosa et al., 2012; USEPA, 2009). In urban watersheds, rainfall events generate stormwater runoff that collects silt, nutrients, metals, microorganisms and other pollutants that accumulate during dry periods, and then affect the water bodies adjacent to urban areas (Makepeace et al., 1995). Traditional municipal stormwater infrastructure conveys contaminants to nearby surface waters with little or no treatment. Therefore, receiving water bodies were impaired by agricultural and urban stormwater runoff in the US (National Research Council, 2009; Steinman et al., 2015; Staley et al., 2018).

Particularly, pathogens are a top pollutant in stormwater associated with impairment of rivers, streams, bays, and estuaries (Ahmed et al., 2019; Jiang et al., 2015; Zhang et al., 2016). Studies addressing the microbial composition of municipal separate storm sewer systems (MS4s) have focused on receiving waters, with few determining the microbial composition of stormwater discharge (Fisher et al., 2015; Mallin et al., 2009; Paule-Mercado et al., 2016). Other studies have evaluated MS4 discharge directly, examining fecal indicator bacteria (FIB) and select human pathogens (Sidhu et al., 2012; Selvakumar and Borst, 2006). However, the relationship between enteric pathogens that cause human illness and the presence of FIBs can be highly variable (USEPA, 2012). Specifically, in surface water impacted by urban runoff, studies have demonstrated a lack of correlation between the presence of *E. coli* and the occurrence of specific enteropathogens (Hörman et al., 2004; Selvakumar & Borst., 2006). In addition, MS4 discharges have exhibited a poor correlation between *E. coli* abundance and enteric pathogen densities (Selvakumar and Borst, 2006). Fisher et al. (2015) utilized high throughput sequencing technology to describe the complete bacterial species profile of MS4 discharge. However, further characterization of the entire MS4 microbial community—including bacteria, fungi, and protists—has yet to be completed, and is important to providing a more comprehensive understanding of the MS4 microbial community and its potential impact on public health.

Moreover, exposure to contaminated surface water used for drinking and recreation sources is linked to adverse health outcomes. A study of 548 incidences of drinking water related disease outbreaks in the U.S. found approximately half resulted in gastrointestinal illness and the other half to be caused by 35 different disease agents (Curriero et al., 2001). Nichols et al. (2009) concludes heavy rainfall precedes many outbreaks related to drinking water, implicating wet weather runoff. A review spanning 1971–1994 found extreme precipitation to be associated with 24% of disease outbreaks associated with raw water derived from reservoir and lakes (Rose et al., 2000). Stormwater-derived microorganisms have been shown to contribute to the contamination of downstream recreational and source waters (Steele et al., 2018). Exposure to marine water impacted by urban runoff increases the risk for skin rash, upper respiratory infection, and gastrointestinal disease symptoms, with increasing risk following storm events and near stormwater outfalls (Tseng and Jiang, 2012; Haile et al., 1999).

In addition to pathogen transmission, stormwater may enable the transmission of antibiotic resistant bacteria and antibiotic resistant genes (Garner et al., 2017). The spread of antibiotic resistance genes (ARGs) is a critical global health threat (Mills and Lee, 2019; World Health Organization, 2015). This can occur in stormwater where bacteria can acquire ARGs in natural matrices through transmission between live cells or by assimilation from the extracellular environment. Studies document urban derived loading of select ARGs in receiving waters originating from sanitary sewers overflow, combined sewer overflows, and MS4s outfalls following rainfall events (Ahmed et al., 2018; Garner et al., 2017; McLellan et al., 2007; Zhang et al., 2016). Garner et al. (2017) characterized the resistome (i.e. complete set of ARGs) in a stream receiving urban stormwater discharge from a majority urban (84%), but partially agricultural (13%) watershed revealing a diverse pool of ARGs that increased in absolute abundance during storm events. However, the resistome of MS4 discharge has yet to be directly investigated and is critical to identifying sources and distribution methods of environmental ARGs, and planning methods to abate their spread.

As part of a larger effort to determine the effect of engineered controls on urban stormwater quality and public health implications, the objectives of this study were to; (1) link *E. coli* levels to rainfall characteristics; (2) identify major fecal contamination sources using microbial source tracking; and (3) establish a baseline microbial contamination profile with a metagenomic approach to examine the microbiome, antibiotic resistance, and virulence factors from stormwater outfalls in Columbus, Ohio, USA.

2. Materials and methods

2.1. Study sites

Four adjacent urban sewersheds and respective sample sites within the Clintonville neighborhood of Columbus, Ohio, USA were selected for study: Beechwold (BW), Indian Springs (IS), Cooke-Glenmont (CG), and Blenheim (BL) (Fig. 1, Table S1). The CG sewershed consists entirely of residential land use, while BW and BL are 96 and 89% residential, respectively (Table 1). Institutional land use (schools, libraries, and community centers) made up <1% and 6.5% of BW and BL. In terms of land use, IS was somewhat different from the other three sewersheds: 75% residential, 8% commercial, and 17% institutional. Businesses within all commercial zones were generally small to moderate in size, and retail oriented. BW (1.12 km^2) had nearly double the drainage area of the next largest sewershed (BL) and CG was the smallest at 0.12 km^2. BW, BL, IS, and CG were drained with 1.37-m diameter, 0.9 by 0.9 m^2, 1.07-m diameter, and 0.46-m diameter concrete outfalls, respectively; Soils in the sewersheds were primarily mapped as silt loams in the Cardington and Bennington soil series.

Impervious surfaces cause reduced infiltration and evapotranspiration in a watershed and are key factors in determining quantity (Shuster et al., 2005) and quality (Carle et al., 2005) of stormwater runoff and the subsequent health of receiving water bodies (Schiff et al., 2007) (Table S1). Imperviousness within the four monitored sewersheds ranged from a minimum of 30.9% (CG) to a maximum of 44.6% (BL). Roofs (40%), roads (20–30%), and driveways (15–25%) represented most of the total impervious area (TIA) in all four sewersheds. Because the downspouts and driveways in Clintonville often directly discharge to the street, the majority of the TIA was directly connected to the sewer system. Pervious areas were primarily residential yards and sporting fields; few natural or forested areas existed in the

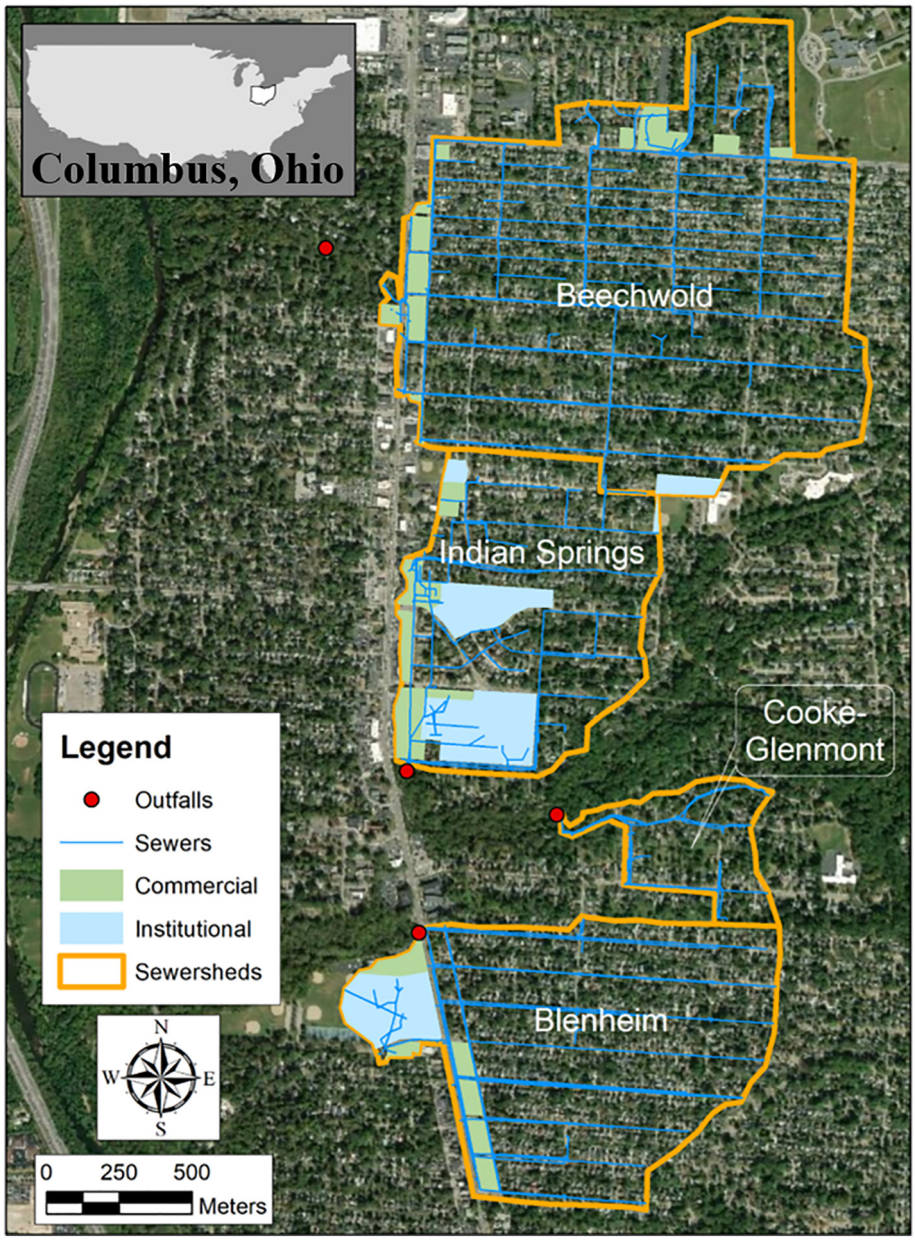

Fig. 1. The four adjacent urban watersheds and sample sites were in the Clintonville area of Columbus, Ohio: Beechwold (BW), Indian Springs (IS), Cooke-Glenmont (CG), and Blenheim-Glencoe (BL). Commercial and institutional land uses are shown in blue and green polygons, respectively. All remaining land within each sewershed was residential. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

sewersheds. As this neighborhood was developed primarily in 1910–1940, few stormwater controls existed in the sewersheds. All sewers in the neighborhood are separated, conveying stormwater and wastewater in different pipes.

Table 1
The four adjacent urban watersheds and sample sites were in the Clintonville area of Columbus, Ohio: Beechwold (BW), Indian Springs (IS), Cooke-Glenmont (CG), and Blenheim-Glencoe (BL).

Sewershed	Area (km^2)	Land Use (km^2)		
		Residential	Commercial	Institutional
Beechwold	1.11	1.07 (95.7%)	0.04 (3.6%)	0.01 (0.7%)
Indian Springs	0.48	0.36 (75.0%)	0.04 (7.6%)	0.08 (17.4%)
Cooke-Glenmont	0.12	0.12 (100%)	0 (0%)	0 (0%)
Blenheim-Glencoe	0.61	0.54 (88.6%)	0.03 (4.9%)	0.04 (6.5%)

2.2. Stormwater sample collection and rainfall data collection

A single rain gauge cluster, consisting of a tipping bucket and a manual rain gauge attached to a 2-m tall wooden post, was located in each of the four experimental sewersheds. Rainfall data were collected using 0.25 mm resolution Davis Rain Collector tipping bucket rain gauges (Davis Instruments, Hayward, CA, USA) and stored on Hobo Pendant data loggers (Onset Computer Corporation, Bourne, MA, USA). Rainfall data were stored on a 1-minute interval and downloaded to a field laptop every third week. Manual rain gauges (Productive Alternatives, Fergus Falls, MN, USA) were checked after each rainfall event for calibrating automated samplers. Rain gauge clusters were installed in locations free from overhead obstructions.

Area velocity sensors (AVM; Teledyne Isco, Lincoln, NE) were utilized to determined flow rate at the four outfalls locations. These sensors measure velocity by emitting a continuous ultrasonic wave to

determine velocity of particles and bubbles in the flow. The frequency shift in the returned signal (i.e. the Doppler Effect) is used to determine the average flow velocity. An internal pressure transducer in the AVM measures flow depth. These measurements along with the known outfall cross-section were used by ISCO 6712 samplers to determine flow rate on a 1-minute interval. Flow rates were integrated with time to determine stormwater volume and trigger sample aliquots. Aliquots were paced such that up to a 50-mm rainfall event could be effectively sampled. Within each sampler, aliquots were composited in a single 20 L bottle and thus characterized pollutant event mean concentrations (EMC). Storm events were separated using the following criteria: a minimum antecedent dry period (ADP) of 6 h and a minimum rainfall depth of 2.5 mm.

Stormwater samples were transferred to the Environmental Microbiology Laboratory, College of Public Health, The Ohio State University. *E. coli* counts were obtained from all sample locations on five days in 2017: 4/11, 6/5, 6/23, 7/21, and 9/5 except for samples absent from CG on 9/5 and 10/23, BL on 6/23 and 10/23, and IS on 6/5. Importantly, the metagenomic data is cross-sectional, derived from four samples (one from each catchment area) obtained on 4/17/2017 only.

2.3. E. coli enumeration

E. coli quantification was performed within 6 h of sample collection utilizing USEPA method 1603, *E. coli* in water by membrane filtration technique (USEPA, 2009). For each sample, three dilutions (1/100, 1/1000, and 1/10000) were prepared in duplicate and filtered through sterile 0.45 μm nitrocellulose membrane filters (47 mm diameter, Millipore Sigma, Burlington, MA, USA). Processed filters were placed on modified mTEC agar (Difco, Detroit, MI, USA) plates and incubated for 2 h at 40 °C followed by approximately 22 h at 37 °C. Red colored *E. coli* colonies were then counted to determine the average number of colony forming units (CFU) per 100 mL.

2.4. DNA extraction

In preparation for DNA extraction, undiluted 100 mL aliquots of each sample were filtered through sterile 0.21 μm membrane filters (Millipore, Burlington, MA, USA) in duplicate. Microbial DNA was extracted from the membrane filters using QIAamp DNA Stool Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Quantification and quality of the extracted DNA were determined using Qubit 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.5. Droplet digital PCR: Microbial source tracking and antibiotic resistance

The presence of PCR inhibitors in the samples was confirmed using Sketa22 assay. Previous study showed that ddPCR measurement was much less affected by PCR inhibition in determining target bacteria than quantitative PCR (qPCR) (Sedlak et al., 2014). In our previous study, we also found the same phenomenon when we compared qPCR and ddPCR in detecting certain bacteria from wastewater and river water (data not shown). Therefore, we chose to use ddPCR that outperforms qPCR in samples that may have high PCR inhibitions, such as stormwater. Microbial source tracking (MST) assays were applied to examine potential sources of fecal contamination. To determine the fecal contamination source, five different host markers were analyzed: (1) UniBac (Universal fecal contamination); (2) Rum2Bac (ruminants); (3) HF183 (human); (4) DogBac (dog); and (5) GFD (avian). The sequences of the primers that were used in quantitative PCR (qPCR) assays were used in our previous studies (Gorham et al., 2017; Healy-Profitós et al., 2016). In addition, three ARGs, including tetracycline resistance gene (*tetQ*), sulfonamide resistance gene (*sul1*), and *Klebsiella pneumoniae* carbapenemase resistance gene (KPC) were measured (Gupta et al., 2019). All experiments were conducted using QX200 droplet digital PCR systems (ddPCR, Bio-Rad, Hercules, CA, USA). For the quantification of UniBac, Rum2Bac, HF183, DogBac, GFD, and KPC, the probe-based ddPCR was used. The total volume of each reagent was 20 μL containing DNA template, 10 μL ddPCR Supermix for probe Mix, nuclease-free water, 250 nM primers, and 250 nM probe. SYBR ddPCR analysis was applied for quantification of *sul1* and KPC genes. The total volume of each reagent was 20 μL containing DNA template, 10 μL ddPCR EvaGreen Supermix, nuclease-free water, 250 nM primers, and 250 nM probe. After mixing the PCR reagents, the droplets were generated using QX200 droplet generator (Bio-Rad). The PCR cycling methods followed our previous studies, with an initial cycle at 50 °C for 2 min, then 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 30s, and, lastly, annealing the extraction under proper conditions followed by reference conditions (Gorham et al., 2017; Gupta et al., 2019; Healy-Profitós et al., 2016). After PCR amplification, droplets were analyzed using the Droplet Reader and QuantaSoft software version 1.7 (Bio-Rad).

2.6. Whole genome shotgun metagenomic analysis

DNA samples for metagenomic analysis were prepared and then sequenced using an Illumina HiSeq (Connelly et al., 2019). Unassembled metagenomic sequencing reads were directly analyzed by CosmosID bioinformatics software package (CosmosID Inc., Rockville, MD, USA) described elsewhere (Hasan et al., 2014; Lax et al., 2014; Ottesen et al., 2016, Ponnusamy et al., 2016) to achieve identification at the species, subspecies, and/or strain level. Each organism's relative abundance was also quantified. Briefly, the system utilizes curated genome databases comprising 65,000 microbial genomes and gene sequences, and a high performance data-mining algorithm that rapidly disambiguates hundreds of millions of metagenomic sequence reads into the discrete microorganisms engendering the particular sequences. The performance of the system has been validated using a large number of datasets including in silico, laboratory constructed, and orthogonally validated biological samples derived from various sources and sample types. Similarly, virulence factors of bacterial community and the resistome, the collection of ARGs in the microbiome, were determined through systemic interrogation of unassembled sequence reads against the CosmosID curated antibiotic resistance gene database.

2.7. Statistical analysis

All statistical analyses were performed using JMP software (V14.0.1; SAS Institute, Cary, NC). A one-way ANOVA test was performed to determine the statistical significance of mean *E. coli* concentrations among locations. A bivariate analysis was conducted of *E. coli* vs. rainfall depth and *E. coli* vs. mean rainfall intensity. The associated lines of best fit were log (base e)-transformed, and a Pearson correlation analysis was used to determine relationships. To determine significance, all statistical testing utilized alpha level of 0.05. Relative abundance and the Shannon-Weiner Diversity Index–having demonstrated superior results assessing the metagenomes of prokaryotic organisms–were calculated using Microsoft Excel 2016 (Release ver. 16.0.9029.2253) (Chernov et al., 2015).

3. Results

3.1. Fecal bacteria abundance and rainfall

Descriptive *E. coli* and rainfall statistics were summarized by sample location (Table 2, Fig. S1). Among the four sample locations, mean *E. coli* abundance was not significantly different ($F = 0.8324$, $p = .4996$). Total *E. coli* mean was 3.22×10^5 CFU/100 mL across the four locations. Relative to rainfall depth, *E. coli* concentration varied widely at low depth, coalescing below the total mean within an increasingly narrow range toward higher depth, but no significant relationship emerged ($r = -0.287$, $p = .264$) (Fig. 2a). In contrast, *E. coli* was positively related

Table 2
Descriptive statistics of *E. coli* abundance and rainfall.

	E. coli (CFU/100 mL)					Rainfall	
Sample Location	BW	IS	CG	BL	Total	Mean Intensity (in./h)	Total Depth (in.)
Sample #	$n = 5$	$n = 4$	$n = 4$	$n = 4$	$n = 17$	$n = 17$	$n = 17$
Minimum	9.0×10^3	2.0×10^3	1.0×10^4	1.0×10^4	2.0×10^3	0.01	0.10
Maximum	6.0×10^5	1.0×10^6	3.0×10^6	7.0×10^4	3.0×10^7	0.67	2.44
Range	5.9×10^5	1.0×10^6	3.0×10^6	6.0×10^4	3.0×10^6	0.66	2.34
Mean	1.6×10^5	3.3×10^5	8.1×10^5	3.5×10^4	3.2×10^5	0.19	0.69
SD	2.5×10^5	4.6×10^5	1.5×10^5	3.0×10^4	7.4×10^5	0.20	0.64

BW: Beechwold, IS: Indian Springs, CG: Cooke-Glenmont, BL: Blenheim-Glencoe.

to mean rainfall intensity (rainfall depth/event duration) ($r = 0.661$; $p = .004$; 95% C.I. = 0.264, 0.866) (Fig. 2b).

3.2. Microbial source tracking and ARGs

The MST results showed that the majority of the stormwater outfalls were positive for ruminant fecal contamination (91% positive for Rum2Bac), ranging from 1.6×10^3 to 7.2×10^3 gene copies/100 mL (Fig. 3, Table 3). Bird fecal contamination was identified at IS, BL, CG (45% positive), ranging from 2.8×10^2 to 3.2×10^3 gene copies/100 mL. Canine fecal contamination was identified in IS and CG (64% positive), ranging from 2.8×10^2 to 4.2×10^3 gene copies/100 mL, and human fecal contamination was detected in BW, IS, and BL (73% positive), ranging from 2.0×10^2 to 2.2×10^3. The mean concentrations of total fecal contamination measured by UniBac was 4.6×10^4 gene copies/100 mL, ranging from 6.8×10^3 to 8.6×10^4 gene copies/100 mL. In addition, the concentration of universal fecal contamination was significantly different between sample location ($p < .05$).

Antibiotic resistance genes were found in the samples (Fig. 4, Table 3). The mean concentration in the samples of the *tet*Q (3.1×10^3 gene copies/100 mL) was about ten times higher than that of *sul*1 (4.3×10^2 gene copies/100 mL) and KPC (2.4×10^2 gene copies/100 mL). The concentration of *tet*Q was significantly different between sample locations ($p < .05$), but the KPC concentration was not significantly different between sample locations ($p > .05$).

3.3. Microbial community: relative abundance and diversity

The sequencing depth of samples ranged between 42.926 and 55.388 million reads (average: 48.608). Of the total population, the bacterial community was dominated by the phyla *Proteobacteria* (55.8%) and *Actinobacteria* (35.9%) with *Thaumarchaeota* (2.9%), *Bacteroidetes* (2.6%), *Firmicutes* (1.7%), and others (<1%) representing lesser relative abundance. *Proteobacteria* was most frequently found in BW and CG, while *Actinobacteria* dominated IS and BL (Fig. 5a). The families *Pseudomonaceae*, *Solirubrobacteraceae*, *Nocardioidaceae*, *Nitrososphaeraceae*, *Geodermatophilaceae*, *Flavobacteriaceae*, *Bradyrhizobiaceae* *Xanthomonadaceae*, *Streptomycetaceae*, *Oxalobacteraceae*, *Comamonadaceae* and *Mycobacteriaceae* each represented >5% of the total relative abundance. Distinctly, *Pseudomonaceae* accounted for 29.2% abundance relative to other families in BW, while the other locations did not exhibit similar single-family dominance.

Fungal species *Alternaria alternate*, *Aureobasidium pullulans*, *Clavaria fumosa*, *Enterocytozoon bieneusi*, *Melampsora pinitorqua*, Onygenales (unclassified), *Puccinia arachidis*, *Talaromyces piceae*, *Thermomyces lanuginosus*, and *Scedosporium apiospermum* each represented >5% of the total relative abundance within at least one location. *C. fumosa*, *M. pinitorqua*, *P. arachidis*, unclassified Tricholomatacea, and the unclassified order Onygenales were present in all samples. Unclassified Onygenales was found most frequently in all locations (Fig. 5b).

Protist species *Acanthamoeba palestinensis, Hammondia hammondi, Naegleria fowleri, Pseudoperonospora cubensis,* and *Reticulomyxa filose* together represent the majority relative abundance in all four samples. The remaining protist species include *Acanthamoeba mauritaniensis, Paramecium biaurelia, Paramecium caudatum, Plasmodium ovale,* unclassified order Oomycetes, *Stylonychia lemnae,* unclassified genus *Thalassiosira*, and *Trypanosoma congolense* (Fig. 5c).

Fig. 5d demonstrates the Chao diversity indices of bacterial, fungal, and protist communities in each location. Diversity varied marginally by location within each microbial taxon (Fig. 5d). Overall, bacteria exhibited greater species diversity (mean = 95, [69, 139]) than fungi (mean = 12, [6.0, 17.5]) and protists (mean = 6.8, [6.0, 8.3]). The Chao diversity index in BW was the highest and CG was the second.

3.4. Virulence genes and resistome

Each sewershed contained a unique profile of ARGs. In total, 17 ARGs were identified, yet none were present in every sample (Fig. 6). In general, ARG distribution within and among samples varied widely. Aminoglycoside resistant genes exhibited high relative abundance in samples

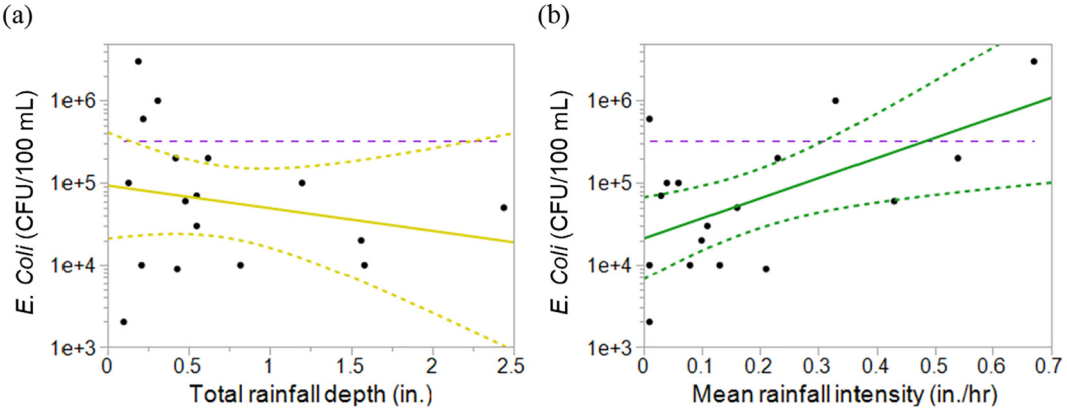

Fig. 2. Relationship between *E. coli* abundance and total rainfall depth ($r = -0.287$, $p = .264$) (a) and between *E. coli* abundance and mean rainfall intensity ($r = 0.661$, $p = .004$) (b). Each graph contains a total *E. coli* abundance mean fit line and natural log transformed line of best fit with 95% confidence boundaries.

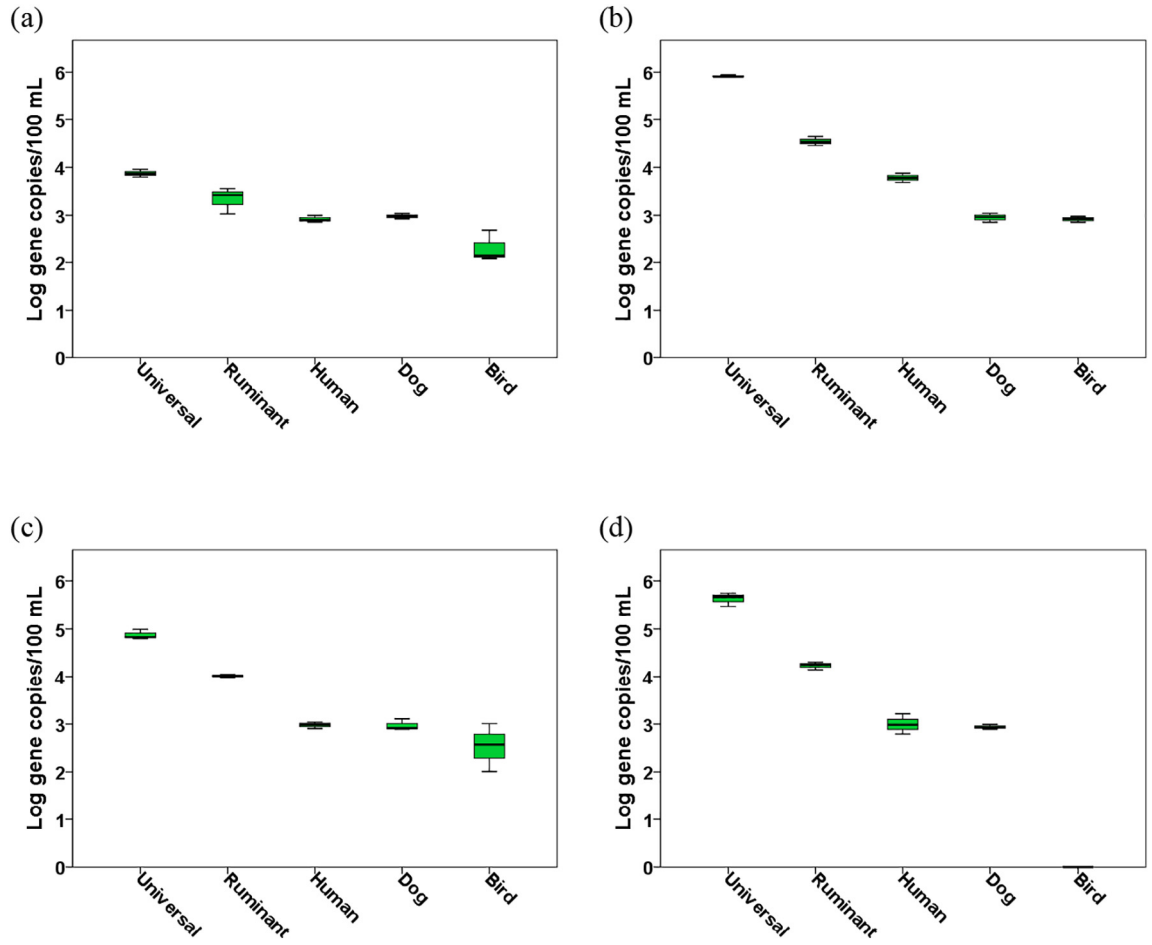

Fig. 3. Boxplots for the results of MST marker concentrations at Beechwold (a), Indian Springs (b), Cooke-Glenmont (c), and Blenheim (d). The ends of the whiskers represent the minimum and maximum concentrations, the bottom and top of the box are the 1st and 3rd quartiles, and the line within the box is the median.

at BW (76.9%) and BL (59.8%); multidrug resistance gene Efflux-pump mepA (37.6%) and chloramphenicol resistance gene Phenicol dha 1 (29.8%) were greatest at CG; and multidrug resistant Efflux-pump sav 1866 (33.4%) was greatest at IS. The most prevalent genes among samples were beta-lactam resistance genes, detected in all four samples, while aminoglycoside and tetracycline resistant genes were detected in BW, IS, and BL.

Virulence genes enable a pathogen to subvert or elude hosts' defenses. Eight virulence genes and their associated host pathogen species were identified (Fig. 7 and Table S3). *Staphylococcus aureus* was most predominant and *Burkholderia pseudomallei* was also present at all locations, *Enterobacter aerogenes* and *Pseudomonas aeruginosa* were present in three locations, *Kleibsiella pneumoniae* in two locations, while *Escherichia coli* and *Salmonella enterica* Typhimurium were found in one location. BW contained the highest number of human pathogen species (*Burkholderia pseudomallei*, *Enterobacter aerogenes*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella* Typhimurium, *Staphylococcus aureus*) in contrast to BL, which contained the lowest (*Burkholderia pseudomallei*).

Table 3
Summary of the results (mean ± standard deviation) of microbial source tracking (universal, ruminant, human, dog, and bird fecal contamination) and antibiotic resistance genes (tetracycline, *tetQ*; sulfonamide, *sul1*; and carbapenemase, KPC) at each sampling location. Units are $\log_{10}$ gene copies/100mL. Differences between sampling locations of each targeted gene were analyzed by one-way ANOVA with all pairwise comparisons using Tukey's HSD. The means marked with the same letter are not significantly different.

	BW	IS	CG	BL
Microbial source tracking				
Universal	3.88 ± 0.08 [a]	5.92 ± 0.03 [b]	4.87 ± 0.10 [c]	5.62 ± 0.15 [d]
Ruminant	3.33 ± 0.27 [a]	4.55 ± 0.10 [b]	4.02 ± 0.03 [c]	4.23 ± 0.08 [a,c]
Human	2.92 ± 0.07 [a]	3.78 ± 0.10 [b]	2.98 ± 0.07 [a]	3.00 ± 0.22 [a]
Dog	2.98 ± 0.06 [a]	2.95 ± 0.10 [a]	2.97 ± 0.12 [a]	2.94 ± 0.05 [a]
Bird	2.30 ± 0.32 [a]	2.92 ± 0.07 [a]	2.53 ± 0.50 [a]	n.d.
Antibiotic resistance gene				
tetQ	2.62 ± 0.02 [a]	4.31 ± 0.15 [b]	3.48 ± 0.14 [c]	3.28 ± 0.03 [c]
sul1	2.41 ± 0.47 [a,b]	2.60 ± 0.30 [a,b]	3.32 ± 0.39 [a]	2.22 ± 0.28 [b]
KPC	2.50 ± 0.37 [a]	2.48 ± 0.38 [a]	2.42 ± 0.33 [a]	3.02 ± 0.56 [a]

BW: Beechwold, IS: Indian Springs, CG: Cooke-Glenmont, BL: Blenheim-Glencoe, n.d.: not detected.

4. Discussion

Human activities in urban areas generate significant volumes of biological and chemical pollutants that can be transported to near surface water. In this study, we revealed quality of urban stormwater, microbial communities, and potential public health implications.

The observed mean total *E. coli* concentration (7.38×10^5 CFU/100 mL) is generally greater than concentrations reported in the National Stormwater Quality Database (Maestre and Pitt, 2005) and is two orders of magnitude greater than other storm event-related MS4 *E. coli* concentrations previously reported; i.e. 3.54 CFU/100 mL (Sidhu et al., 2012), 5.77×10^3 MPN/100 mL (Parker et al., 2010), 2.56×10^4 MPN/100 mL (Hathaway et al., 2010) and 1.5–8.5 10^3 CFU/100 mL (Selvakumar and Borst, 2006). No significant difference of *E. coli* concentration across locations may be attributed to similar climatic, land use, and age of development conditions. Ecosystem structure, species composition, soil composition, materials of the built environment, etc. are assumed to be likely equipollent. Previous studies have also shown that *E. coli* abundance does not vary with land use (high and low density

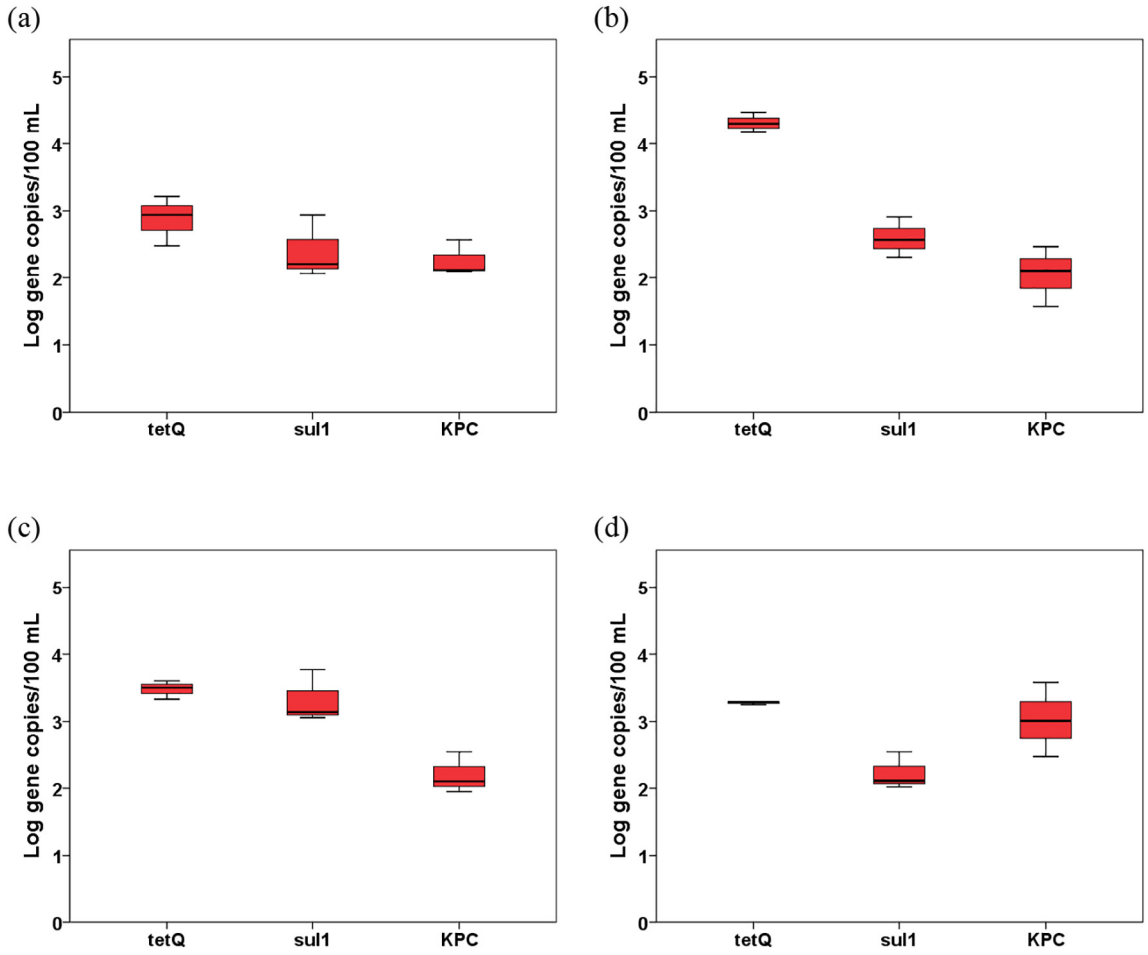

Fig. 4. Boxplots for antibiotic resistance gene concentrations (tetracycline (*tet*Q), sulfonamide (*sul*1), and carbapenem resistance (KPC)) using ddPCR at Beechwold (a), Indian Springs (b), Cooke-Glenmont (c), and Blenheim (d). The ends of the whiskers represent the minimum and maximum, the bottom and top of the box are the 1st and 3rd quartiles, and the line within the box is the median.

residential, and commercial) but do vary by season, with highest densities in the spring and summer (Selvakumar and Borst, 2006).

Despite the lack of significant correlation between *E. coli* concentration and rainfall depth, the trend indicates as rainfall depth increases *E. coli* concentration decrease to a range below the total mean. Furthermore, *E. coli* densities did significantly increase with intensifying rainfall (rainfall depth/duration). These results suggest intense, short duration rainfalls yield the highest fecal contamination in stormwater. These findings are consistent with other reports (McCarthy et al., 2007). This may be related to the suspension of greater amounts of sediment during high intensity rainfall events. Therefore, urban runoff may be a potential source of pathogen impairment in source waters.

We also considered major fecal contamination sources of urban stormwater. "Ruminant" sources are likely White-Tailed Deer (Cervidae), the only wild ruminant in Ohio (Ohio Department of Natural Resources, 2012). Deer are frequently observed in the residential sewersheds. Deer are a reservoir for many zoonotic diseases (Kruse et al., 2004); among them, virulence genes of *Salmonella* Typhimurium were reported. Dog and bird fecal contamination was commonly detected in stormwater in Rotterdam, the Netherlands (Sales-Ortells and Medema, 2015). Interestingly, detection of human-fecal contamination potentially implies cross contamination from a leaking sanitary sewer, an illicit connection of sanitary waste to the storm sewer, or other unidentified sources of infiltration (Steele et al., 2018).

We highlight microbial species related with potential health implications to exposed individuals. Therefore, the microbial community, including bacteria, fungi, and protists, with particular interest in antibiotic resistance and virulence genes, was investigated in the stormwater. The dominant families (>5% relative abundance) contain versatile environmental organisms affiliated with diverse habitats and often associated with water. Their sources include soil, stone, aerosols, vegetation, wild and domestic macro-organisms, and humans.

Especially, the results of virulence factors demonstrate a potential public health risk from urban stormwater outfalls. The majority of virulence genes were identified within the less abundant families (<1% relative abundance). We identified virulence genes of *P. aeruginosa* that are a major agent of nosocomial infection known to cause wound, burn, and urinary tract infections (Palleroni, 1981). Soil-dwelling *Burkholderia pseudomallei* causes Melioidosis, a pulmonary infection and significant cause of mortality in southeast Asia and northern Australia. A variety of animals are susceptible to melioidosis, including cattle, pigs, deer, cats, and dogs. (Cheng and Currie, 2005). *Enterobacter aerogenes* and *Klebsiella pneumoniae* are commonly found in soil and surface water, rarely infect healthy individuals, and are problematic hospital-acquired infections (Prince et al., 1997). Pathogenic strains of *E. coli* are a major cause of gastrointestinal foodborne illness in the U.S., often associated with contaminated water which is a source for the transmission of this pathogen (Daniels et al., 2000). In addition, waterborne outbreaks have been documented related with *Salmonella enterica* serovar Typhimurium which is the second most common *Salmonella* serovar to cause salmonellosis (Bonetta et al., 2011). *Staphylococcus aureus* is commonly found on the skin and upper respiratory tract of healthy humans and animals. Related illnesses included food

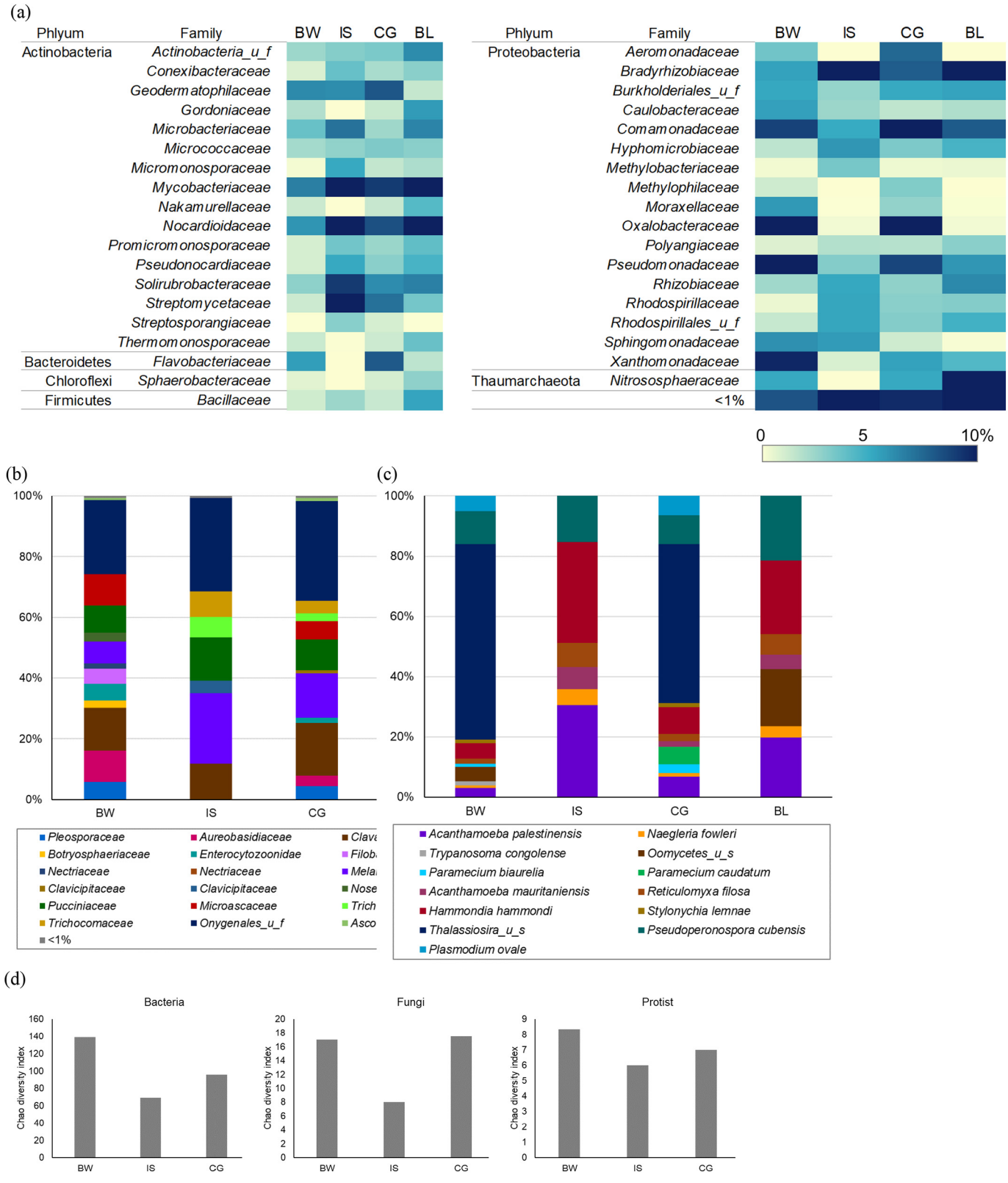

Fig. 5. Relative abundance of bacteria at phylum and family levels (a), fungi at species level (b), protist at species level (c) by sample location. Chao diversity index for bacteria, fungi, and protists at each sample location (d). BW: Beechwold, IS: Indian Springs, CG: Cooke-Glenmont, BL: Blenheim-Glencoe.

poisoning (via toxin) and skin disorders. Notorious for antibiotic resistance, *S. aureus* is one of the most common healthcare-associated infections (Klevens et al., 2007).

Protists can serve as indicators of water quality and contaminant levels (Jiang and Shen, 2007). However, broad studies of protists in urban environments are scarce. In this study, the protist community was profiled, including water molds and Oomycetes (unclassified); plant pathogen *Pseudoperonospora cubensis*; paramecium *P. biaurelia* and *P. caudatum*; cat and mouse parasite *Hammondia hammondi* which has never been shown to infect humans (Frenkel and Dubey,

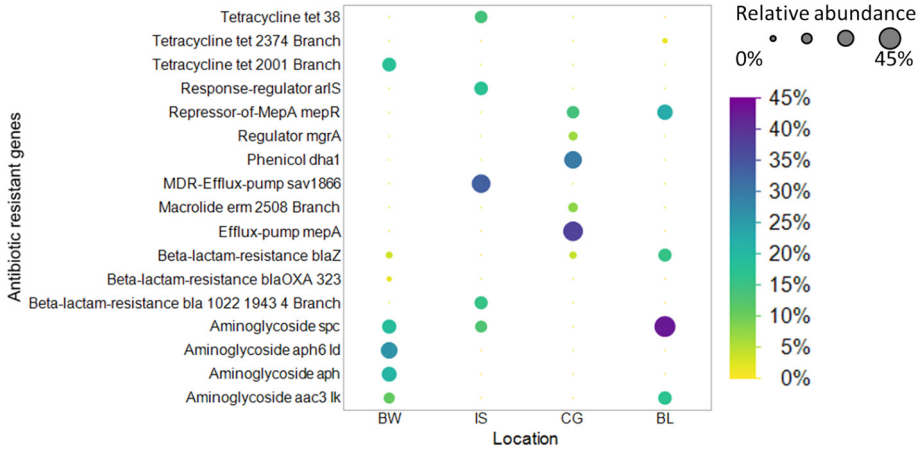

Fig. 6. Relative abundance of antibiotic resistance genes by sample location (BW: Beechwold, IS: Indian Springs, CG: Cooke-Glenmont, BL: Blenheim-Glencoe). The size and color of circles are for relative abundance of antibiotic resistance genes.

1975); and *Reticulomyxa filose*, a foraminiferan amoeboid that lives in terrestrial freshwater habitats (Ostwald and Hülsmann, 1988). Some taxa of public health importance were detected. *Acanthamoeba palestinensis* and *Acanthamoeba mauritaniensis* are causative agents of granulomatous amebic encephalitis and amebic keratitis, respectively. *A. palestinensis* infects the central nervous system, mostly affecting

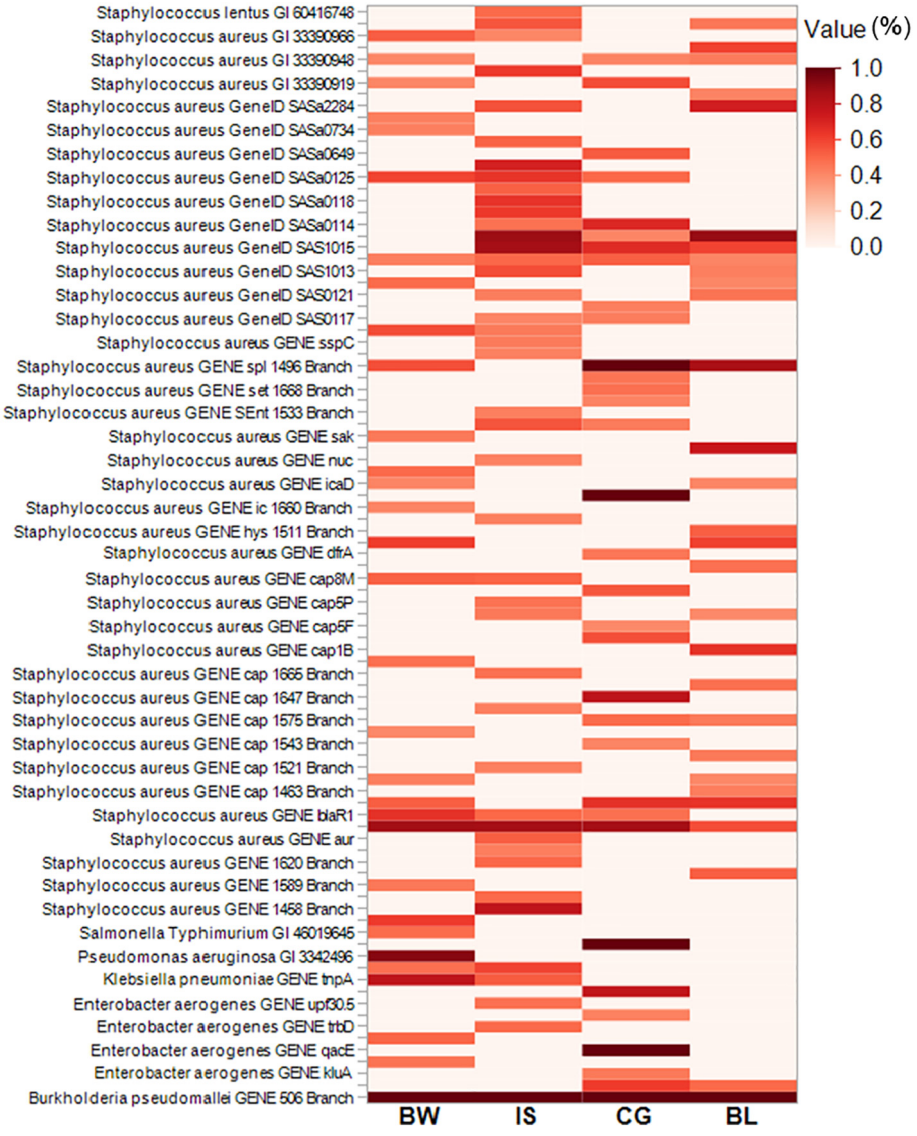

Fig. 7. Bacterial species associated with virulence genes by location. BW: Beechwold, IS: Indian Springs, CG: Cooke-Glenmont, BL: Blenheim-Glencoe.

immunocompromised individuals (Parija et al., 2015). *A. mauritaniensis* infects the cornea (outer eye covering). People who swim while wearing contact lenses are at greater risk for the disease (Lorenzo-Morales et al., 2015). In addition, *Naegleria fowleri,* which is a thermophilic free-living amoeba found in soil and warm freshwater environments, causes a rare but fatal and central nervous system disease, primary amebic meningoencephalitis (Grace et al., 2015). Although several studies reported *N. flowleri* contamination in man-made and natural water bodies (swimming pools, ponds, hot springs, and hot tubs) and soil, infection with *N. fowleri* has been not linked to the *N. fowleri* detection (Grace et al., 2015; Maclean et al., 2004). Infections predominately occur in recreational lakes and ponds during the summer months in warm weather locations, such as south eastern parts of the US (Yoder et al., 2010). A small proportion of *Plasmodium ovale* was identified in BW and CG samples, which is one of two closely related protozoan species that cause *Plasmodium ovale* malaria in humans (Fuehrer and Noedl, 2014; Collins and Jeffery, 2005). *P. ovale* infections are considered relatively mild and easily curable with common antimalarials (Mueller et al., 2007; Rojo-Marcos et al., 2011). The presence of *P. ovale* has been reported throughout the world (Collins and Jeffery, 2005). Although eliminated from the United States, locally transmitted mosquito-borne (all type) malaria outbreaks have occurred, the most recent in 2003. In 2014, 5.2% of 1724 U.S. malaria cases were identified as *P. ovale* (Mace, 2017). Franklin county, where Columbus is located, accounts for approximately half of all malaria cases in Ohio and contains seven endemic *Anopheles* species (Ohio Department of Health, 2020). Of them, *An. quadrimaculatus* is the only proven vector of *P. ovale* and one of the two dominant endemic species (Jeffery, 1954; Ohio Department of Health, personal communication, November 4th, 2018).

The most dominant fungal species (>5% relative abundance) were plant pathogens or saprophytes, such as *Alternaria alternate*, *Aureobasidium pullulans*, *Clavaria fumosa*, *Melampsora pinitorqua*, *Puccinia arachidis*, and *Thermomyces lanuginosus* (Mattila, 2005; Mondal and Badigannavar, 2015; Singh et al., 2003; Zalar et al., 2008). Two of the dominant fungal species cause invasive fungal disease in immunocompromised human. *Scedosporium apiospermum,* is an environmental mold widespread in soil, sewage, and polluted waters. Infection of the sinuses, lungs, and skin may occur (Goldman et al., 2016). *Enterocytozoon bieneusi* infects intestinal cells of humans, livestock, cats, and other mammals (Matos et al., 2012).

In this study, the patterns of ARG compositions in urban stormwater were examined. Mapping potential sources and transport mechanisms of ARGs in the environment can help in design of management strategies aimed at reducing their dissemination and potential incorporation by pathogenic bacteria. These MS4 outflows were shown to contain a variable ARG profile with mobile genetic elements. Our findings imply that stormwater may be a potential source of ARGs to receiving water environments.

A major limitation of this study is that the metagenomic data was limited to only a subset of the single day samples from each study site. It is unlikely to have provided a complete catalogue of the microbiome, resistome, or microbial source data. Future study could with enhanced sample can strengthen these profiles by examining a temporal dataset across multiple seasons and storm events.

CRediT authorship contribution statement

Seungjun Lee: Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization. **Michael Suits:** Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization. **David Witusynski:** Investigation, Data curation, Writing - review & editing. **Ryan Winston:** Data curation, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Jay Martin:** Writing - review & editing, Supervision, Project administration, Funding acquisition. **Jiyoung Lee:** Conceptualization, Methodology, Resources, Writing - original draft, Supervision, Project administration, Funding acquisition.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.138033.

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