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Stormwater loadings of antibiotic resistance genes in an urban stream

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ABSTRACT

Antibiotic resistance presents a critical public health challenge and the transmission of antibiotic resistance via environmental pathways continues to gain attention. Factors driving the spread of antibiotic resistance genes (ARGs) in surface water and sources of ARGs in urban stormwater have not been well-characterized. In this study, five ARGs (*sul*1, *sul*2, *tet*(O), *tet*(W), and *erm*(F)) were quantified throughout the duration of three storm runoff events in an urban inland stream. Storm loads of all five ARGs were significantly greater than during equivalent background periods. Neither fecal indicator bacteria measured (*E. coli* or enterococci) was significantly correlated with *sul*1, *sul*2, or *erm*(F), regardless of whether ARG concentration was absolute or normalized to 16S rRNA levels. Both *E. coli* and enterococci were correlated with the tetracycline resistance genes, *tet*(O) and *tet*(W). Next-generation shotgun metagenomic sequencing was conducted to more thoroughly characterize the resistome (i.e., full complement of ARGs) and profile the occurrence of all ARGs described in current databases in storm runoff in order to inform future watershed monitoring and management. Between 37 and 121 different ARGs were detected in each stream sample, though the ARG profiles differed among storms. This study establishes that storm-driven transport of ARGs comprises a considerable fraction of overall downstream loadings and broadly characterizes the urban stormwater resistome to identify potential marker ARGs indicative of impact.

1. Introduction

The World Health Organization has deemed the emergence and spread of antibiotic resistance a crisis that "threatens the very core of modern medicine" (World Health Organization, 2015). Though concerns first centered on nosocomial patterns of resistance, the potential role of environmental pathways in facilitating the spread of antibiotic resistance among bacteria has gained considerable attention. Multiple studies have documented the contamination of surface waters with antibiotic resistance genes (ARGs) originating from wastewater treatment plants (Garcia-Armisen et al., 2011; Graham et al., 2011; Munir et al., 2011), agricultural runoff (Chee-Sanford et al., 2009; Fahrenfeld et al., 2014; Joy et al., 2013), and urban stormwater (McLellan et al., 2007; Zhang et al., 2016). Though numerous studies have documented increased loadings of pathogens and fecal indicator bacteria to surface water following rainfall (Hathaway and Hunt, 2010; Liao et al., 2015; McCarthy et al., 2012; Sidhu et al., 2012; Surbeck et al., 2006), potentially associated increases in loadings of antibiotic resistant bacteria and their associated ARGs have not been considered.

Given that soil bacteria represent a natural reservoir of ARGs, simple detection in environmental matrices is not necessarily of concern. However, point and non-point source pollution can serve as anthropogenic sources of ARGs to the environment (Pruden et al., 2012), thus the potential for dissemination of ARGs to waterborne and/or opportunistic environmental pathogens via horizontal gene transfer calls for consideration. The ability of bacteria to acquire ARGs horizontally between live cells (conjugation), via bacteriophage infection (transduction), or via assimilation from the extracellular environment (natural transformation) necessitates consideration of the total abundance of ARGs in an

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environmental sample (i.e., the resistome) (von Wintersdorff et al., 2016). The potential risk of transfer of extracellular ARGs and ARGs carried by non-pathogenic bacteria to pathogens in aquatic environments is largely uncharacterized at this point. For example, recent work demonstrated that the plasmid-mediated colistin resistance gene MCR-1 can easily pass between strains of *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* (Liu et al., 2016), which are common intestinal/environmental species. Consequently, tracking resistance risks requires inclusion not only of known pathogens or microorganisms currently expressing resistance, but also resistance encoding genetic material that may be incorporated by pathogens.

Surface water has been identified as a reservoir of diverse and even novel ARGs (Amos et al., 2014a; Bengtsson-Palme et al., 2014; Garner et al., 2016; Kristiansson et al., 2011; Port et al., 2012). Stormwater in particular possesses many characteristics that may lead to the selection and amplification of these genes. Stormwater can be contaminated from an array of point and non-point sources, including land-applied manure, septic tanks, combined sewer overflows and leaky sewers (Kelsey et al., 2004; Parker et al., 2010; Sauer et al., 2011). It also frequently contains substantial quantities of heavy metals (Sansalone and Buchberger, 1997) and antibiotics (Davis et al., 2006; Joy et al., 2013; Xu et al., 2013), which are both well-known to select bacteria that possess ARGs, even at sub-inhibitory concentrations (Andersson and Hughes, 2014; Gullberg et al., 2014; Liu et al., 2011; McVicker et al., 2014). Sub-inhibitory concentrations are of great interest given that they are generally more environmentally-relevant, but also because “inhibitory” concentrations are only defined in limited strain, and/or media-specific contexts. At low levels, heavy metals and antibiotics have also been observed to stimulate horizontal gene transfer (Beaber et al., 2004; Klümper et al., 2016; Prudhomme et al., 2006; Song et al., 2009; Úbeda et al., 2005; Xia et al., 2008; Zhang et al., 2013), increasing the potential for resistant native aquatic bacteria to transfer ARGs to pathogenic bacteria introduced by stormwater.

While urban stormwater and associated runoff have been thoroughly documented as a source of pathogens (Cizek et al., 2008; Qureshi, 1979; Selvakumar and Borst, 2006; Sidhu et al., 2012), the role of storms in propagating ARGs has received little attention. Although patterns of incidence have been examined in several watersheds to date (Amos et al., 2014a; Chen et al., 2013a; Graham et al., 2011; Luo et al., 2010; Marti et al., 2013), the sources and mechanisms contributing to these observations, and their connections to various anthropogenic inputs are poorly understood. Thus, the identification of likely sources and transport processes of ARGs during storms represents an important knowledge gap.

The objectives of this study were to: (1) characterize the abundance of five ARGs (two sulfonamide: *sul*1 and *sul*2; two tetracycline: *tet*(O) and *tet*(W); and one macrolide: *erm*(F)) in an inland urban stream throughout the duration of three rainfall events and during baseline conditions; (2) identify physicochemical and hydrometeorological factors related to the occurrence or abundance of ARGs in stormwater runoff; and (3) investigate the breadth of the resistome detectable during storms relative to baseline levels using next-generation high-throughput DNA sequencing. Understanding the incidence and movement of ARGs within urban streams will better inform watershed management strategies to mitigate downstream risks.

2. Materials and methods

2.1. Site and storm descriptions

Samples were collected from Stroubles Creek in Blacksburg, Virginia, USA at the Stream Research, Education, and Management Laboratory (StREAM Lab; http://www.bse.vt.edu/site/streamlab/). The Stroubles Creek watershed has been extensively described in previous studies (Liao et al., 2015, 2014; VADEQ VADCR, 2006); the 14.4 km^2 drainage area above the study's sampling point is 84% urban/residential land use (served by municipal sanitary sewers), 13% agricultural land use (primarily pasture and cropland), and 3% forested land. On-site instrumentation stations record a suite of physicochemical variables (temperature, specific conductivity, pH, turbidity, dissolved oxygen) via multiparameter water quality sondes (YSI Inc.) as well as streamflow (stage) via a gauge (Campbell Scientific, Inc.) (Liao et al., 2014).

Samples were collected during three summer storms occurring on June 27, July 2, and July 10, 2013 and are herein referred to as storms 1, 2, and 3, respectively. Rainfall depths of 6, 17, and 12 mm were recorded for the three storms, respectively, and total event runoff volumes were calculated to be 8,100 m^3, 37,000 m^3, and 70,000 m^3 (Liao et al., 2015). Additional hydrometeorological characteristics of the studied storms have been previously published (Liao et al., 2014, 2015).

2.2. Sample collection and DNA extraction

Water samples were collected automatically in sterile 750 mL bottles every 15 (storm 1) or 30 (storms 2 and 3) minutes using a 6712 ISCO sampler (Teledyne, Lincoln, NE) over each storm's duration. Three baseline samples were also collected from the sample site during dry weather periods (e.g., no appreciable precipitation or change in stream stage for the previous 24 h). Samples were transported on ice and stored at 4 °C prior to processing. Within 24 h of collection, samples were thoroughly shaken to mix and 50 mL aliquots were filter-concentrated onto 0.4 μm pore size polycarbonate membrane filters (Millipore, Billerica, MA). Filters were transferred to 2 mL sterile tubes and stored at −80 °C. Filters were cut into approximately 1 cm^2 fragments using a flame-sterilized blade and transferred to DNA extraction tubes. DNA was extracted from the filters using a PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA) according to manufacturer instructions.

2.3. Molecular analysis and high throughput sequencing

ARGs were quantified in triplicate reactions from DNA extracts using qPCR with previously published protocols for 16S rRNA genes (Suzuki et al., 2000) and five ARGs: *sul*1, *sul*2 (Pei et al., 2006), *tet*(O), *tet*(W) (Aminov et al., 2001), and *erm*(F) (Chen et al., 2007). A subset of samples was initially analyzed at dilutions of 1:10, 1:20, 1:50, and 1:100 to determine the minimum dilution required to minimize inhibition (results not shown); ultimately, a dilution factor of 1:10 was selected and applied to all extracts. Triplicate standard curves of ten-fold serial diluted standards of each target gene ranging from 10^2 to 10^8 gene copies/μl for 16S rRNA and 10^1 to 10^7 gene copies/μl for ARGs were included on each 96-well plate, along with a triplicate negative control. The minimum acceptable qPCR standard curve R^2 was 0.978. The limit of quantification was established as the lowest standard that amplified in triplicate in each run, and was equivalent to 10^4 gene copies per L of sampled bulk water for *sul*1, *sul*2, *tet*(O), *tet*(W), and 16S rRNA genes, and 10^5 gene copies per L of sampled bulk water for *erm*(F).

Shotgun metagenomics were conducted on the sample representing the maximum stream stage (i.e., peak flow) during each storm, as well a composite of the three baseline samples, combined by equal DNA mass. Samples were prepared using the Nextera XT library preparation (Illumina, San Diego, CA) and sequenced on an Illumina HiSeq 2500 using a 100-cycle paired-end protocol at the

Biocomplexity Institute of Virginia Tech Genomics Research Lab. Paired end reads were merged using FLASH (Magoč and Salzberg, 2011). Quality filtering was conducted using Trimmomatic (Bolger et al., 2014) according to default parameters. 16S rRNA genes were annotated using BLASTN (Altschul et al., 1997) against the GreenGenes ribosomal RNA database (DeSantis et al., 2006). ARGs were annotated using the DIAMOND protein aligner (Buchfink et al., 2014) against the subset download of the Comprehensive Antibiotic Resistance Database (McArthur et al., 2013) that excludes genes that confer resistance via specific mutations (accessed August 2015). A minimum amino acid identity of 90% and a minimum e-value of 10^{-5} were required. Metagenomes were uploaded to the metagenomics RAST server (MG-RAST) (Meyer et al., 2008) and are publicly available under the accession numbers 4628882.3–4628885.3.

2.4. Data analysis

All statistical comparisons were conducted using R (v. 3.2.1) with a significance cutoff of $\alpha = 0.05$ unless otherwise noted. Normality of ARG datasets was assessed using a Shapiro-Wilk test. All ARG datasets failed to meet normality requirements, with the exception of *sul*1, thus non-parametric statistical analyses were applied for all ARG comparisons. ARG abundances during various storm phases were compared using a Kruskal-Wallis rank sum test, followed by a pairwise Wilcoxon rank sum test. Spearman's rank correlation coefficients were calculated to assess correlations between ARG abundances, fecal indicator bacteria, water quality parameters, and hydrometeorological parameters. Storm ARG event loads (EL; gene copies/event) and equivalent background period loads (EBP; gene copies/EBP) were calculated as previously described for fecal indicator bacteria (Liao et al., 2015):

$$EL = \sum_{i=1}^{N} Q_i C_i \Delta t \quad (1)$$

$$EBP = EL/_{DL} \quad (2)$$

where Q_i = *i*th discrete discharge (L/s); C_i = *i*th discrete ARG concentration (gene copies/L); Δt = sampling interval (s); N = number of discrete samples collected; and DL = mean dry-weather loads in the duration of the storm event (gene copies). Event ARG loads were compared to equivalent background period loads using a Wilcoxon rank sum test.

3. Results and discussion

3.1. Selection of ARG targets for characterizing storm loadings

Five ARGs representing three classes of resistance (two sulfonamide: *sul*1 and *sul*2; two tetracycline: *tet*(O) and *tet*(W); and one macrolide: *erm*(F)) were selected for quantification in all samples via qPCR to characterize how loads of ARGs change throughout the course of each storm (Fig. 1). The five target ARGs were selected due to their documented prevalence in watersheds impacted by anthropogenic and agricultural runoff (Fahrenfeld et al., 2014; Pruden et al., 2006; Storteboom et al., 2010a). Macrolides and tetracyclines are among the most widely used antibiotics globally for both human and agricultural applications (Van Boeckel et al., 2014). Sulfonamides were the first synthetically produced antibiotic for widespread use beginning in the 1930s and extensive resistance has since emerged among clinical isolates (Sköld, 2000). *sul*1 and *sul*2 have been widely detected in municipal wastewater (Laht et al., 2014; Munir et al., 2011; Pruden et al., 2012) and *sul*1 is of particular interest as it has been identified as a strong indicator of anthropogenic influence in surface water (Pruden et al., 2012). *tet*(O) and *tet*(W) have been commonly found in wastewater, but are also common targets found in agricultural waste streams (Auerbach et al., 2007; Koike et al., 2007; McKinney et al., 2010). Additionally, all five of these genes were among those identified as candidate indicators of the extent of the impact of antibiotic resistance in the environment by the European Cooperation in Science and Technology Action (Berendonk et al., 2015).

3.2. Gene loading rates and intra-storm variability

Absolute concentrations of sulfonamide ARGs responded most consistently during storm events, with *sul*1 elevated above baseline concentrations during storms 1 and 3 ($p = 0.0070$; 0.0060), and *sul*2 was significantly elevated above baseline concentrations during all three storms ($p = 0.0141$; 0.0122; 0.0077). In contrast, tetracycline and macrolide ARGs were not consistently elevated, with *tet*(W) only significantly greater than the baseline during storm 2 ($p = 0.1412$) and *erm*(F) during storm 3 ($p = 0.0493$). Absolute concentrations of *tet*(O) were not significantly different from baseline concentrations during any storms. Total 16S rRNA genes were significantly elevated across all storms ($p = 0.0070$; 0.0110; 0.0060).

ARG concentrations were also compared among defined portions of each storm: rising limb, peak flow, falling limb, and established baseline (Fig. 2). While absolute abundances are important for determining total loading of ARGs, changes in the abundance of total bacteria can obscure patterns of ARG enrichment with respect to the overall microbial community. Therefore, both absolute (gene copies/L) and relative (gene copies/16S rRNA gene copies) abundances are considered. During storm 1, the absolute concentration (gene copies per L) of *sul*1, *sul*2, *tet*(W), and 16S rRNA genes all trended higher during the peak and rising limb of the storm compared to the baseline, but were only significantly greater during the rising limb ($p = 0.0119$; 0.0138; 0.0138; 0.0119). In terms of relative abundance, only *tet*(W) and *erm*(F) were greater during the rising limb ($p = 0.0138$; 0.0138), while *sul*2 fell below the baseline ($p = 0.0138$) (Fig. 2). In contrast, during storm 2, only absolute concentrations of *sul*2 and 16S rRNA genes were significantly elevated relative to the baseline during the falling limb of the storm ($p = 0.0053$; 0.0030). Notably, absolute concentrations of *sul*2 trended above the baseline by greater than 1-log during all phases of the storm. During storm 3, the absolute concentration of all five ARGs and 16S rRNA genes were elevated above the baseline during the falling limb ($p \leq 0.0140$), which is a remarkable contrast to storm 1, where concentrations were elevated during the rising limb. *sul*2 again trended above the baseline on average during each phase of the storm, by greater than 1.5-log. 16S rRNA genes were elevated above the baseline during each phase of the storm by greater than 1-log.

The unique profile of the five target ARGs that occurred during various phases of the storm suggest point and non-point sources of ARGs may vary both over the duration of each storm and among individual storm events. The non-uniform concentration of ARGs throughout each storm suggests that certain urban and agricultural sources influenced the sampling point at different times throughout the sampling scheme. This is similar to the observation that fecal indicator bacteria in general can exhibit large variations during intra-storm sampling, with greater than 0.5-log variation in *E. coli* and enterococci concentrations documented during a single storm (Liao et al., 2015; Stumpf et al., 2010). The apparent ubiquity of *sul*1 in Stroubles Creek suggests that it is not introduced to the watershed exclusively during times of rainfall, which may be attributed to a number of unique characteristic the gene possesses.

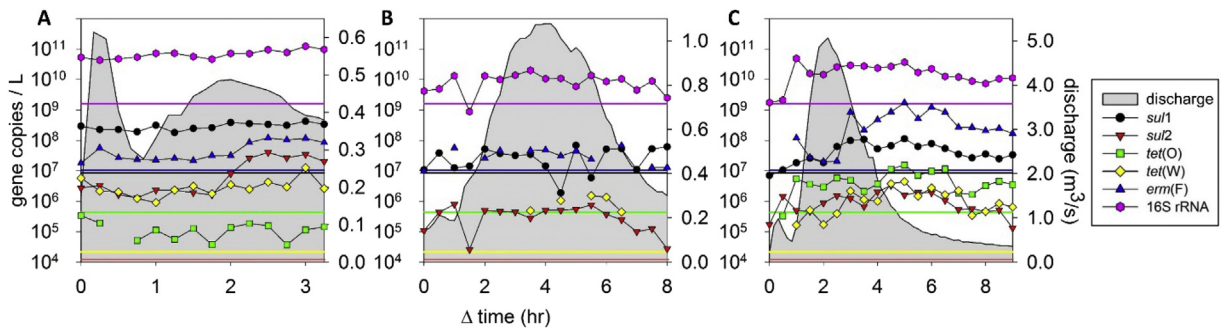

Fig. 1. ARG abundance with respect to Stroubles Creek discharge. Absolute abundances of ARGs and 16S rRNA genes (gene copies/L; left axis) and stream discharge (m^3/s; right axis) for (A) storm 1, (B) storm 2, and (C) storm 3.

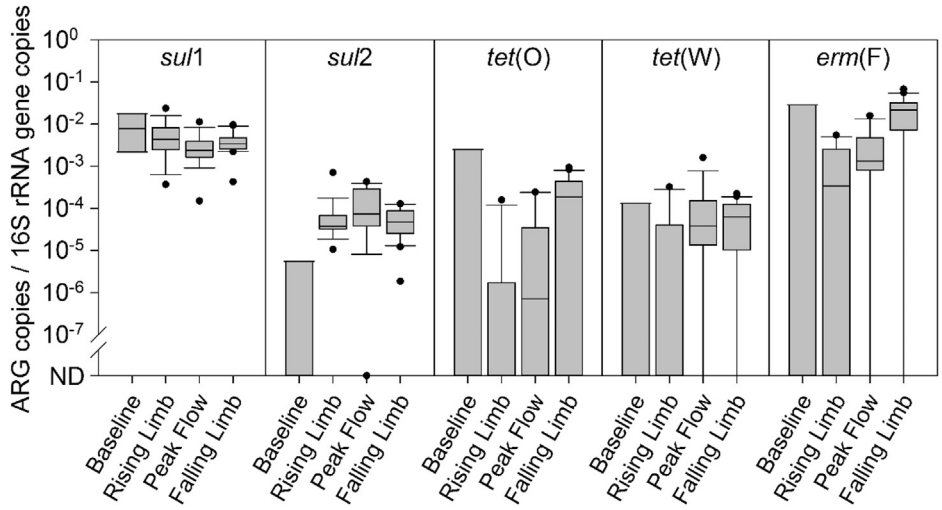

Fig. 2. ARG relative abundances during storm phases. Relative abundances of ARGs (ARG copies/16S rRNA gene copies) during baseline sample collection, storm rising limb, storm crest, and storm falling limb across all storms.

*sul*1 is widely associated with plasmids and transposons, making it prone to horizontal gene transfer and common in both pathogens and environmental bacteria (Sköld, 2000). Additionally, *sul*1 tends to reside adjacent to the class 1 integron and a variable number of additional antibiotic ARGs, enabling selection not only by sulfonamide antibiotics, but other antibiotics as well (Huovinen et al., 1995; Mazel, 2006).

If ARG sources leading to the greatest watershed loading of ARGs can be identified, specific watershed management strategies could be identified to limit the long-term propagation of ARGs in watersheds. To explore this possibility, pollutographs were constructed presenting the cumulative gene copy loading versus the cumulative runoff volume. Of particular interest was whether ARGs follow a "first flush" pattern, commonly defined as the transport of 80% of a pollutant loading within the first 30% of a storm's discharge volume (Bertrand-Krajewski et al., 1998) (Fig. 3). Across all three storms, none of the five ARGs exhibited a "first flush" pattern. Rather, all ARGs tend to fall below the 1:1 bi-sector of the pollutographs, indicating that the bulk of ARG loading tended to occur in the latter half of each storm discharge volume. This trend was particularly pronounced for *sul*2 during storm 1, *tet*(W) during all three storms, and *erm*(F) during storms 1 and 3. Previous work indicates that fecal indicator bacteria do not always follow a traditional first flush pattern (Krometis et al., 2007; Stumpf et al., 2010), however, the observed "lag" in ARG transport reported in this study is unique.

3.3. Event loading rates

Total event loads for ARGs and 16S rRNA genes were calculated for each storm and compared to the equivalent background period loading that would occur at baseline concentrations for the equivalent duration of a storm (Fig. 4). Loading of 16S rRNA genes during storm events averaged almost 2-log greater than during the equivalent background period. Similarly, the storm event loads were significantly greater than the equivalent background period loading for all genes, except *tet*(O) (Wilcoxon Rank Sum test; $\alpha = 0.10$; $p = 0.1000$; 0.0765; 0.0765; 0.0765; and 0.1000 for *sul*1, *sul*2, *tet*(W), *erm*(F) and 16S genes, respectively). On average, the total load of each ARG across a storm event was greater than 1-log above the equivalent background load. This increased loading was most dramatic in the cases of *sul*2 and *tet*(W), which each increased at least 2-log above the equivalent background loading for each storm. Total bacterial DNA markers also increased during this time by greater than 1-log in all cases. This increased event loading is critical because, despite the relatively short storm duration (i.e., a few hours of precipitation) there is real potential for lasting surface water quality impacts. For example, once ARGs enter the watershed environment, they are subject to a number of complex fate and transport mechanisms by which they may persist or propagate throughout the aquatic environment via bulk water or by partitioning to sediments (Pruden et al., 2012). ARGs may be transferred to or taken up by native aquatic bacteria, augmenting reservoirs of

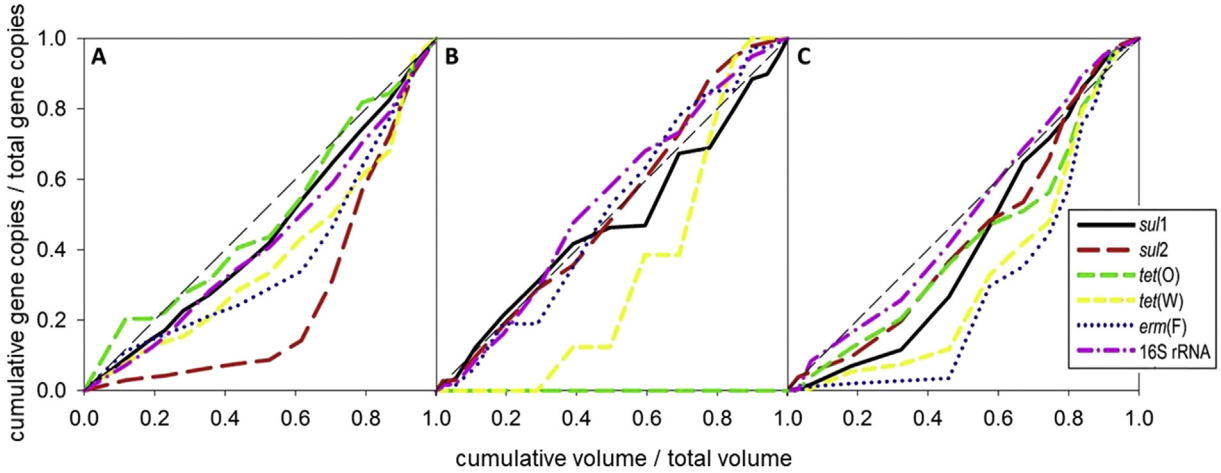

Fig. 3. Cumulative loading distributions for (A) storm 1, (B) storm 2, and (C) storm 3 indicating the cumulative fraction of ARGs with respect to the cumulative runoff volume at the collection point. The dashed 1:1 reference bi-sector indicates a constant absolute (genes per L) concentration of ARGs.

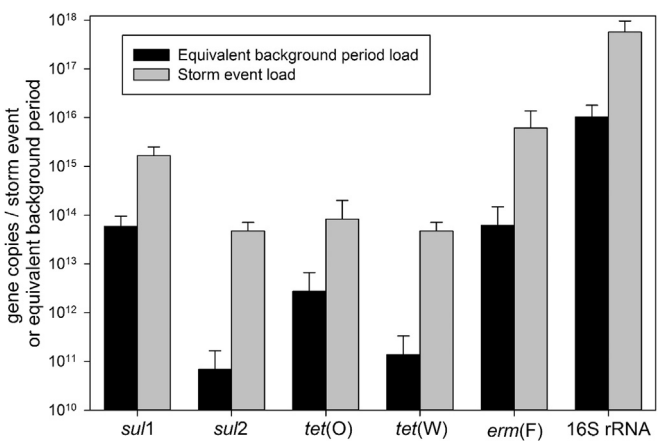

Fig. 4. Average ARG storm event loading and corresponding equivalent background period loading. Error bars represent standard deviation of the gene load of the storms (n = 3) or the equivalent background periods (n = 3).

resistance that have the potential to be subsequently transferred to pathogenic bacteria (Forsberg et al., 2012; Wright, 2010). Residual antibiotics and metals can create selective pressure for ARGs. Studies have also suggested that the presence of pesticides and herbicides can select for bacteria possessing ARGs (Bordas et al., 1997; Kurenbach et al., 2015).

Although no significant correlations were identified between total ARG load and hydrometeorological characteristics of the storms (event rainfall depth, event duration, time to peak flow, and event runoff volume), a few patterns are worth noting. *sul*1 was present at the greatest absolute abundances during storm 1, the storm with the shortest duration (7 h) and least runoff volume (8100 m^3). *sul*1 was present at markedly consistent relative concentrations throughout all storm and baseline samples (Fig. 2), suggesting that *sul*1 is present in Stroubles Creek during various meteorological conditions and subject to dilution under intense storm conditions. By contrast, *tet*(O) and *erm*(F) reached highest absolute concentrations during storm 3. Storm 3 was characterized by the greatest duration (23 h), a relatively short time to peak flow (3 h), and the highest event runoff volume (70,000 m^3), suggesting that *tet*(O) and *erm*(F) are mobilized from contaminant sources under periods of high runoff volume.

3.4. Association with fecal indicator bacteria and environmental variables

Monitoring of fecal indicator bacteria, such as *E. coli* and enterococci, is widely used in regulatory monitoring as a proxy for probable fecal pathogen contamination and associated human health risk. Culturable *E. coli* and enterococci concentrations have previously been published for the storms of interest (Liao et al., 2015). Weak correlations existed between several of the monitored ARGs and fecal indicator bacteria (Table 1). *E. coli* concentrations correlated significantly with absolute (Spearman's $\rho = 0.3627$; $p = 0.0026$) and relative concentrations of *tet*(O) ($\rho = 0.3411$; $p = 0.0047$). *E. coli* also correlated with absolute abundances of *tet*(W) ($\rho = 0.3301$; $p = 0.0064$). Enterococci correlated weakly, but significantly, with absolute abundances of *sul*2 ($\rho = 0.2436$; $p = 0.0488$), *tet*(W) ($\rho = 0.3208$; $p = 0.0086$), and *erm*(F) ($\rho = 0.3144$; $p = 0.0101$). Enterococci exhibited strong significant correlations with absolute *tet*(O) ($\rho = 0.5218$; $p < 0.0001$) concentrations and relative *tet*(O) ($\rho = 0.4753$; $p < 0.0001$). Enterococci also significantly correlated with concentrations of 16S rRNA genes ($\rho = 0.2505$; $p = 0.0425$). These results suggest that fecal indicator bacteria are not consistently an accurate proxy for ARGs resulting from stormwater runoff. *E. coli*, in particular, was not well suited as an indicator for sources of the monitored sulfonamide or macrolide ARGs. Enterococci appear to be a more accurate indicator of contamination by all of the monitored genes, with the exception of *sul*1. Interestingly, *tet*(O) and *tet*(W) were the only ARGs significantly correlated with *E. coli* and the genes with the strongest correlation to enterococci, suggesting that they are likely to be associated with fecal contamination. This is consistent with previous studies that have found *tet*(O) and *tet*(W) to be abundant in environments impacted by swine waste streams, dairy manure-treated agricultural soils, and beef, swine, and dairy waste lagoons (Fahrenfeld et al., 2014; Koike et al., 2007; McKinney et al., 2010). *tet*(O) and *tet*(W) are known to be carried by a relatively diverse range of bacterial hosts, having been previously identified in at least 20 and 25 genera, respectively, including both Gram-positive and -negative bacteria as well as both pathogens and environmental bacteria (Roberts, 2011). Both genes may be carried chromosomally or within conjugative plasmids, and have been associated with mobile elements, such as transposons (Chopra and Roberts, 2001; Roberts, 2012). Enterococci have been known to carry *tet*(O) while neither *E. coli* nor enterococci typically carry

Table 1
Spearman's rank correlation coefficients indicating correlations between ARGs, fecal indicator bacteria, and physicochemical water quality parameters. Statistically significant correlations ($\alpha = 0.05$) indicated in bold and with an asterisk.

	E. coli	enterococci	temperature	turbidity	dissolved oxygen	conductivity	pH
*sul*1	0.123	0.167	**0.279***	**0.392***	−0.188	−0.206	**−0.285***
*sul*2	0.165	**0.244***	**0.327***	**0.467***	−0.221	−0.165	**−0.251***
tet(O)	**0.363***	**0.522***	**0.446***	**0.753***	**−0.397***	**0.316***	−0.063
tet(W)	**0.330***	**0.321***	**0.312***	**0.542***	**−0.266***	−0.138	**−0.274***
erm(F)	0.150	**0.314***	**0.324***	**0.619***	**−0.508***	**0.271***	**−0.339***

tet(W) (Chopra and Roberts, 2001), suggesting that while *tet*(W) may be associated with other fecal-associated bacteria, the correlations are likely not due to direct carriage by *E. coli* or enterococci.

Stream water temperature also correlated significantly with absolute abundances of all ARGs, as well as total 16S rRNA genes (R = 0.2790; 0.3272; 0.4460; 0.3116; 0.3242; 0.2795; p = 0.0222; 0.0069; 0.0002; 0.0103; 0.0074; 0.0220 for *sul*1, *sul*2, *tet*(O), *tet*(W), *erm*(F), and 16S genes respectively). Elevated temperatures in urban stormwater are often associated with runoff from impervious surfaces (Jones et al., 2012) and fecal sources of contamination (Paule-Mercado et al., 2016). Turbidity was also significantly correlated with absolute concentrations of all ARGs and 16S rRNA genes (R = 0.3915; 0.4671; 0.7525; 0.5417; 0.6187; 0.4904; p = 0.0011; <0.0001; <0.0001; <0.0001; <0.0001; <0.0001 for *sul*1, *sul*2, *tet*(O), *tet*(W), *erm*(F), and 16S genes respectively). Though elevated turbidity may be associated with fecal contamination in freshwater streams, stream bed sediment disturbance as well as particulate matter in runoff from impervious surfaces can also contribute significantly to elevated turbidity. Dissolved oxygen was negatively correlated with absolute concentrations of *tet*(O), *tet*(W), and *erm*(F) (R = −0.3967; −0.2663; −0.5084; p = 0.0009; 0.0294; <0.0001). Deficient dissolved oxygen concentrations are widely associated with urban storm runoff (Keefer et al., 1980), suggesting that it is a source of input to Stroubles Creek for these genes.

3.5. Diversity and richness of the resistome

While *sul*1, *sul*2, *tet*(O), *tet*(W), and *erm*(F) are all well-documented as frequently detected in surface waters impacted by agricultural runoff and treated wastewater, application of next-generation sequencing technologies to samples collected from similar environments have revealed the presence of diverse ARGs beyond these key genes of interest. Therefore, we applied shotgun metagenomic sequencing to a subset of samples to gain insight into the broader resistome present during peak storm conditions as compared to baseline conditions in order to inform ARG selection for future surface water monitoring efforts. Shotgun metagenomic high-throughput DNA sequencing produced 13.6–18.4 million paired 100-bp reads per sample. Between 409 and 1157 reads per sample (0.003–0.009% of reads) were identified as probable ARG sequences via annotation against the Comprehensive Antibiotic Resistance Database (McArthur et al., 2013). Abundances of ARGs are presented normalized to abundance of 16S rRNA genes, as well as target gene length and 16S rRNA gene length as described previously (Li et al., 2015). Normalized abundance of total ARGs ranged from 0.17 to 0.30 ARGs per 16S rRNA gene. A total of 162 different ARG were annotated across the dataset, with 57, 37, 100, and 121 ARGs annotated in the baseline sample and storms 1, 2, and 3, respectively (Table S1). Across the dataset, trimethoprim was the most abundant class of antibiotic resistance (35.8% of total ARGs), followed by multidrug (33.8%), beta-lactam (6.8%), polymyxin (6.7%), aminoglycoside (5.6%), and glycopeptide resistance (3.1%) (Fig. 5). As many as 155 ARGs have been detected in a single sample in previous studies, as well as ARGs capable of conferring resistance to all major classes of antibiotics (Amos et al., 2014b; Bengtsson-Palme et al., 2014; Chen et al., 2016, 2013b; Garner et al., 2016). The relative prevalence of multidrug resistance among detected ARGs is comparable to the findings of other metagenomic studies characterizing ARGs in surface water and associated sediments (Chen et al., 2013a; Garner et al., 2016; Li et al., 2015) and is likely due to the prevalence of multidrug efflux pumps among environmental bacteria (Martinez, 2009). Elevated trimethoprim resistance is less common among comparable metagenomic studies, but trimethoprim ARGs have been detected in environments heavily impacted by aquaculture and agriculture (Byrne-Bailey et al., 2009; Muziasari et al., 2014), making the source of abundant trimethoprim ARGs unclear in this urban watershed.

Notably, multidrug, beta-lactam, peptide, and tetracycline resistance (0.14, 0.030, 0.0083, and 0.0022 ARGs per 16S rRNA genes, respectively) were more prevalent during storm 3 compared to other storms and baseline concentrations. In the baseline sample, however, rifampin, aminocoumarin, fluoroquinolone, and glycopeptide resistance (0.0070, 0.00029, 0.0011, and 0.013 ARGs per 16S rRNA genes, respectively) were more abundant than levels observed during the storms.

Only 14 ARGs were consistently present during the baseline as well as all three storms: one trimethoprim resistance gene (*dfr*E), two polymyxin ARGs (*Pmr*E, *ros*B), one nalidixic acid ARG (*emr*B), and ten genes that are components of multidrug efflux pumps or involved in the modulation of multidrug efflux (*acr*F, *ceo*B, *mdt*B, *mdt*C, *mex*B, *mex*C, *mex*D, *pho*P, *sme*R, *sme*B). Each storm contained a unique profile of ARGs, with 8, 25, and 38 ARGs annotated uniquely to storms 1, 2, and 3, respectively. There was not a conserved ARG profile across the storms; however, ten ARGs were detected in all storms but were absent in baseline samples: two aminoglycoside resistance genes (*aad*A, ANT(2")-Ia), one beta-lactam (OXA-12), one peptide (*bac*A), one polymyxin (*arn*A), and five genes related to multidrug efflux pumps (*bae*S, *mdt*D, *mdt*F, *mdt*L, *pho*Q). These ten ARGs could be considered as targets for future storm monitoring efforts, though ARGs unique to storm events may vary among different watersheds.

While the association of ARGs with common fecal indicator bacteria and physicochemical parameters offers insight into the possible sources of ARG contamination in Stroubles Creek, the tendency of certain ARG patterns to be conserved based upon runoff source could provide the basis for ARG source tracking. Several studies have demonstrated the feasibility of profiling the antibiotic resistance of fecal streptococci to identify likely sources of fecal pollution in surface water and groundwater (Hagedorn et al., 1999; Wiggins, 1996; Wiggins et al., 1999). Though widely used for over a decade, current source-tracking strategies generally focus on the detection of source-specific genetic markers (i.e. library-independent strategies), given the labor-intensive nature of antibiotic resistance profiling. Patterns of occurrence of tetracycline ARGs have been used to identify urban and agricultural sources of ARGs in surface water (Chen et al., 2013a; Storteboom et al., 2010a).

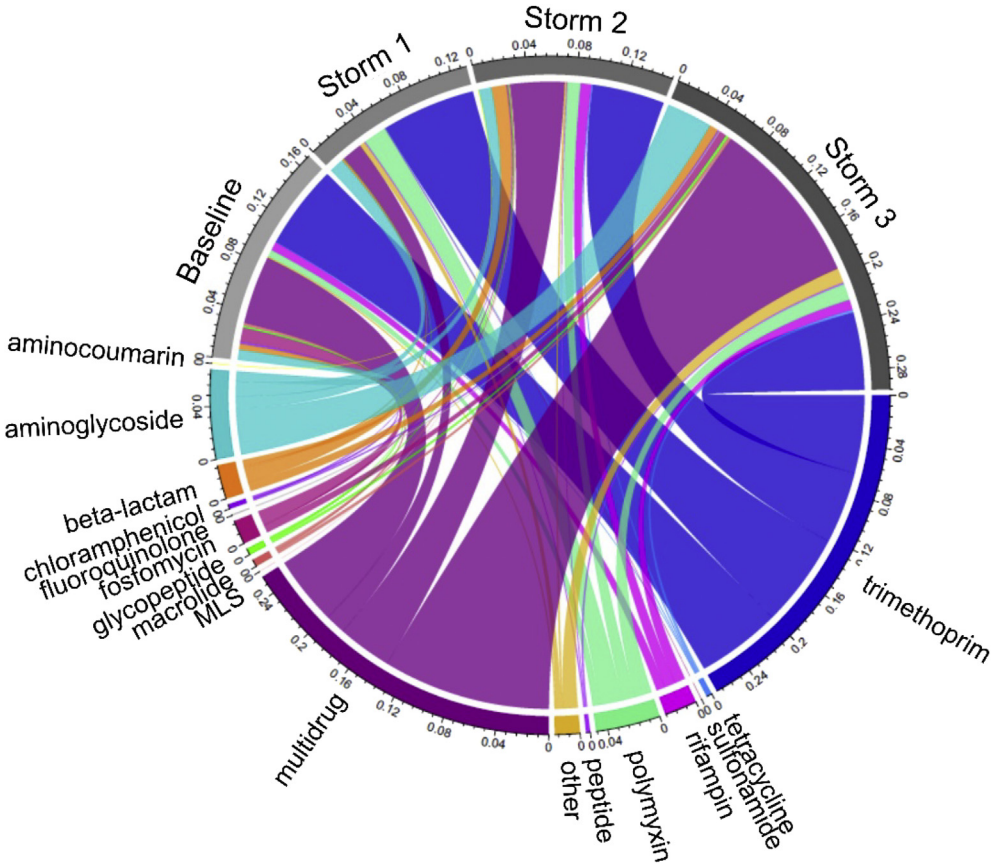

Fig. 5. Distribution of ARGs by class in baseline (composite n = 3) and peak (n = 1) runoff storm samples determined by shotgun metagenomic sequencing. Length of bars around the plot circumference indicate ARG copies normalized to 16S rRNA genes. Figure produced using the circlize package in R (v. 3.2.1).

Storteboom et al. (2010a) demonstrated that certain tetracycline ARGs were more frequently associated with agriculture runoff (*tet*(H), *tet* (Q), *tet* (S), and *tet* (T)), while others were more frequently associated with wastewater treatment plant (WWTP) effluent (*tet*(C), *tet* (E), *tet* (O)). Phylogenetic variations in *tet*(W) have been used to track sources of ARG contamination in groundwater and surface water (Koike et al., 2007; Storteboom et al., 2010b). Storteboom et al. (2010b) utilized restriction fragment length polymorphism analysis to demonstrate that certain *tet*(W) phylotypes were associated with environments impacted by agricultural runoff, while different *tet*(W) phylotypes were indicative of WWTP influence. In future work, such library-independent microbial source tracking methods combined with storm profiling of ARGs could be used to identify waste streams that contribute to the highest watershed loadings of ARGs. Next-generation sequencing offers a powerful tool to be used for examining genetic variation in ARGs and can facilitate the identification of genetic phylotypes associated with specific ARG sources. Management of these ARG sources can help to limit watershed-scale ARG dissemination and potential downstream uptake by pathogenic bacteria.

4. Conclusions

Identification of strategies to limit inputs of clinically-relevant ARGs, along with other initiatives to improve storm water quality, can help alleviate the risk of antibiotic resistance spread. This study tracked the effects of storm events on the loadings of ARGs in an affected stream and provided insight into mechanisms involved in transport as well as the behavior of various indicators of antibiotic resistance. Specific conclusions include the following:

- Storm-driven transport of ARGs contributed significant loadings to surface waters. Loadings of certain ARGs (*sul*2 and *tet*(W)) were more than two orders of magnitude greater during storm conditions than during equivalent background periods.
- Key differences were noted in the behavior of different ARGs during storm runoff, yielding new insight into the processes governing the fate and transport of ARGs in watersheds. For example, the tetracycline resistance genes, *tet*(O) and *tet*(W) were correlated with the fecal indicator bacteria, *E. coli* and enterococci, but *sul*1, *sul*2, and *erm*(F) were not.
- Further research is needed to understand the seasonal and geographic variation in behavior among ARGs in stormwater runoff as well as to identify key “indicator” ARGs or other genetic elements that are associated with risk of downstream transfer to pathogens and antibiotic resistance.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2017.06.046.

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