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Antibiotic resistance genes in an urban stream before and after a state fair

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ABSTRACT

The global spread of antibiotic resistance genes (ARGs) concomitant with a decrease in antibiotic effectiveness is a major public health issue. While research has demonstrated the impact of various urban sources, such as wastewater treatment plant (WWTP) effluent, stormwater runoff, and industrial discharge on ARG abundance in receiving waters, the impact of short-term gatherings such as state fairs is not comprehensively understood. The objective of this research was to explore the impact of a 2-week Wisconsin State Fair gathering – over 1.1 million visitors and 7,100 farm animals – on the abundance of the ARG bla_{TEM}, the integrase of the class 1 integron (*intI1*), a marker for horizontal gene transfer, and the 16S rRNA gene, a marker for total biomass, in an urban stream receiving runoff from the state fair. Stream samples downstream of the state fair were taken before and after the event and quantified via a droplet digital polymerase chain reaction. The absolute abundance of all genes was significantly higher ($p<0.05$) following the event. This research showcases the prevalence and persistence of ARG contamination in an urban stream before and after a state fair gathering, suggesting that short-term events can be a significant source of ARGs into the environment.

Key words: antibiotic resistome, droplet digital PCR, mobile genetic elements, short-term events, urban stormwater runoff

HIGHLIGHTS

- The concentration of three genes was quantified from an urban stream before and after a short-term event which was characterized by the large presence of people and animals.
- Five storms occurred during the short-term event with runoff conveyed from the fairgrounds to the urban stream.
- The absolute abundance of all genes, including one antibiotic resistance gene and one integron, was elevated after the event compared to the before event.

INTRODUCTION

Understanding the dissemination and fate of antibiotic resistance genes (ARGs) in the urban aquatic environment is critical for public health worldwide (Vaz-Moreira *et al.* 2014; Sanganyado & Gwenzi 2019). Environmental input sources such as wastewater discharge from municipal, hospital, and pharmaceutical industry waste streams contribute to ARG dissemination into the environment and serve as possible exposure routes to humans (Lapara *et al.* 2011; Pruden *et al.* 2013; Lamba *et al.* 2017; Gwenzi *et al.* 2020). Lesser studied pathways, notably nonpoint source runoff, are also likely contributors to ARG transmission into the urban water cycle. Current research describes nonpoint source runoff as an area of concern for urban ARG pollution. Indeed, recent studies found that the abundance of ARGs in both receiving surface waters (Garner *et al.* 2017) and storm drain outfalls (Ahmed *et al.* 2018; Baral *et al.* 2018) during wet weather events was elevated above baseline values by as much as two orders of magnitude. Further research documented the abundance of the mobile genetic element *intI1*, an indicator of horizontal gene transfer, during dry and wet weather events, detecting *int1* in 100% of the samples taken, with a maximum concentration of 8.29 $\log_{10}$ gene copies/L (Gillings *et al.* 2015; Ahmed *et al.* 2018). The pollutants found in runoff including ARGs, residual antibiotics, and mobile genetic resistant elements can select for antibiotic resistant bacteria and facilitate gene transfer in receiving surface waters (Berglund 2015; Von Wintersdorff *et al.* 2016). This finding suggests that runoff could be a significant source of environmental antibiotic resistance in the urban environment.

In addition to urban sources contributing to environmental antibiotic resistance, watersheds that contain agricultural land uses may be subject to ARGs from animal manure and agricultural wastewaters abundant in antibiotics and ARGs (Chang *et al.* 2015). The dissemination of these waste streams into the environment can occur by amending fields with manure or through the direct discharge of agriculture wastewater into rivers and lagoons (Cheng *et al.* 2013; Pruden *et al.* 2013; He *et al.* 2016). Subsequent precipitation events can trigger sediment erosion and surface runoff from the waters and lands impacted by agricultural wastes, dispersing ARGs, antibiotics, and resistant genetic elements into the environment (Jechalke *et al.* 2014; Nnadozie & Odume 2019). Indigenous bacterial communities under the selective stress of antibiotic resistance pollution have been shown to readily transfer ARGs to exogenous bacteria, including human commensal bacteria and pathogens (Allen *et al.* 2010; Keen *et al.* 2018), resulting in a public health concern as the continued acquisition of ARGs is reducing the effect of antibiotics on human and animal pathogens (Wellington *et al.* 2013; He *et al.* 2016).

A unique combination of these sources – urban runoff and agricultural waste – occurs in the United States during state fair events, where large numbers of animals are gathered over a period of weeks. During these events, the transport of animal waste from the fair grounds to urban water bodies through runoff can result in a unique increase in pollutants from agricultural waste. To that end, urban streams that are hydrologically connected to state fairs have been shown to elevate the presence of fecal coliforms and *Escherichia coli* after the event takes place (Milwaukee Metropolitan Sewerage District 2006). This is particularly of concern for understanding ARG contamination as fecal coliforms and *E. coli* indicators have been shown to directly correlate with ARG abundance (Sáenz *et al.* 2001; Zaniani *et al.* 2012; Vaz-Moreira *et al.* 2014; Eramo *et al.* 2017; Reynolds *et al.* 2020). Consequently, the increase in bacterial loading observed in urban runoff during state fair events, paired with the increased presence of animals and animal waste, suggests that there may be an abundance of ARGs discharged downstream of the fairgrounds.

To test this hypothesis, this study quantified genetic markers in an urban stream before and after a state fair event. Specifically, the impact of the 2019 Wisconsin State Fair, an annual event over 11 consecutive days that brings over a million people and thousands of animals together at one location, was analyzed. The objective of this research was to quantify the integrase gene of the class 1 integrons, *intI1*, and the ARG, bla_{TEM}, in an urban stream that is hydrologically connected to the Wisconsin State Fair. *intI1* was evaluated because it is indicative of horizontal gene transfer, while bla_{TEM} is a resistance gene that confers resistance to a variety of clinically and agrarian relevant antibiotics, notably penicillin, cephalosporin, and carbapenems (Livermore 1995; Poole 2004; Gillings *et al.* 2015). Samples were collected before and after the fair in an urban stream that receives runoff from the State Fairground and were analyzed via droplet digital polymerase chain reaction (ddPCR). This research revealed that state fair events can serve as conduits of ARGs into urban water cycles.

MATERIALS AND METHODS

Sample location and collection

The Wisconsin State Fair is an annual event that takes place in West Allis, Wisconsin. It brings together Wisconsin and out-of-state residents for a competition of agrarian livestock as well as industrial, craft, textile, and culinary products. The State Fair was selected as an appropriate case study because it is an annual large, short-term event that brings together millions of people, but it also has thousands of animals as well as carnival amusement rides and games, recreational activities, and musical concerts, thus incorporating a wide variety of short-term event activities. In 2019, between August 1st and August 11th, 1.13 million people attended the Wisconsin State Fair (Wisconsin State Fair 2019). Approximately, 1,100 dairy cattle, 500 beef cattle, 1,200 goats, 400 horses, 800 poultry, 800 rabbits, 1,300 sheep, and 1,000 swine are transported to the Wisconsin State Fairground for the event (Wisconsin State Fair 2020). The entirety of the fairgrounds is roughly 190 acres, consisting of numerous facilities, including a bi-level livestock barn (32,000 ft^2), a sheep and goat barn (31,304 ft^2 with 166 stalls), three single-level livestock barns (18,240 ft^2 each with 94–96 stalls), a dairy cattle barn (46,400 ft^2 with 172 stalls), a swine and goat (41,580 ft^2 with 145 stalls), the Case IH coliseum with a show ring (57,672 ft^2), and a warm up ring (10,500 ft^2) (Wisconsin State Fair n.d.). In addition, a map of the stormwater infrastructure installed underneath the state fairgrounds and operated and maintained by the city of West Allis can be found in Supplementary Fig. C1. The map is sourced from the city of West Allis and illustrates the hydraulic connectivity of stormwater mains underneath and around the park that convey stormwater runoff to Honey Creek.

Surface water samples were collected immediately downstream from the State Fairgrounds in the Honey Creek in Milwaukee, WI. Honey Creek is an 8.8-mile-long tributary of the Menomonee River that has faced channel modifications including

lining 7.1 miles of the creek with concrete, leading to considerable habitat and ecological degradation and flooding (Milwaukee Metropolitan Sewerage District 2006). Figure 1 shows the sampling location and its proximity to the fairgrounds, as well as indicates the path of the creek in an enclosed underground concrete-lined conduit that runs underneath the State Fairgrounds. Samples were collected downstream of the fairground and north of I-94 where Honey Creek comes above-ground. One-liter grab samples were collected in triplicate in the center of the stream cross section at a depth of one foot before the fair on July 30th, 2019 and after the fair on August 12th, 2019. Samples were taken at mid-day and the average daily temperature was 68 °F and 74 °F on July 30th and August 12th, respectively (Milwaukee Metropolitan Sewerage District 2020). Samples were transported on ice and stored at 4 °C following collection. Within 24-h of collection, all samples were filtered for subsequent DNA extraction as described the *DNA extraction* section and stored at −20 °C.

Stream discharge

Stream discharge in Honey Creek was recorded downstream by U.S. Geological Survey (USGS) stream gage 04087119 Honey Creek at Wauwatosa, WI, latitude 43°02'38', longitude 88°00'10' (Figure 2). The average daily discharge at the time of the grab samples taken before and after the Wisconsin State Fair was recorded at 0.05 and 0.07 cubic meters per second (m^3/s), respectively. Three separate large storms occurred between the sampling events. The peak 5-min discharges for the storms that took place on August 5th at 5:00 AM, August 5th at 11:25 PM, and August 7th at 11:50 PM were 10.4, 12.9, and 10.2 m^3/s, respectively. Two smaller storms occurred on August 3rd and August 11th, with the August 11th storm notably occurring the day before the second sampling event. Though the magnitude and duration of this storm

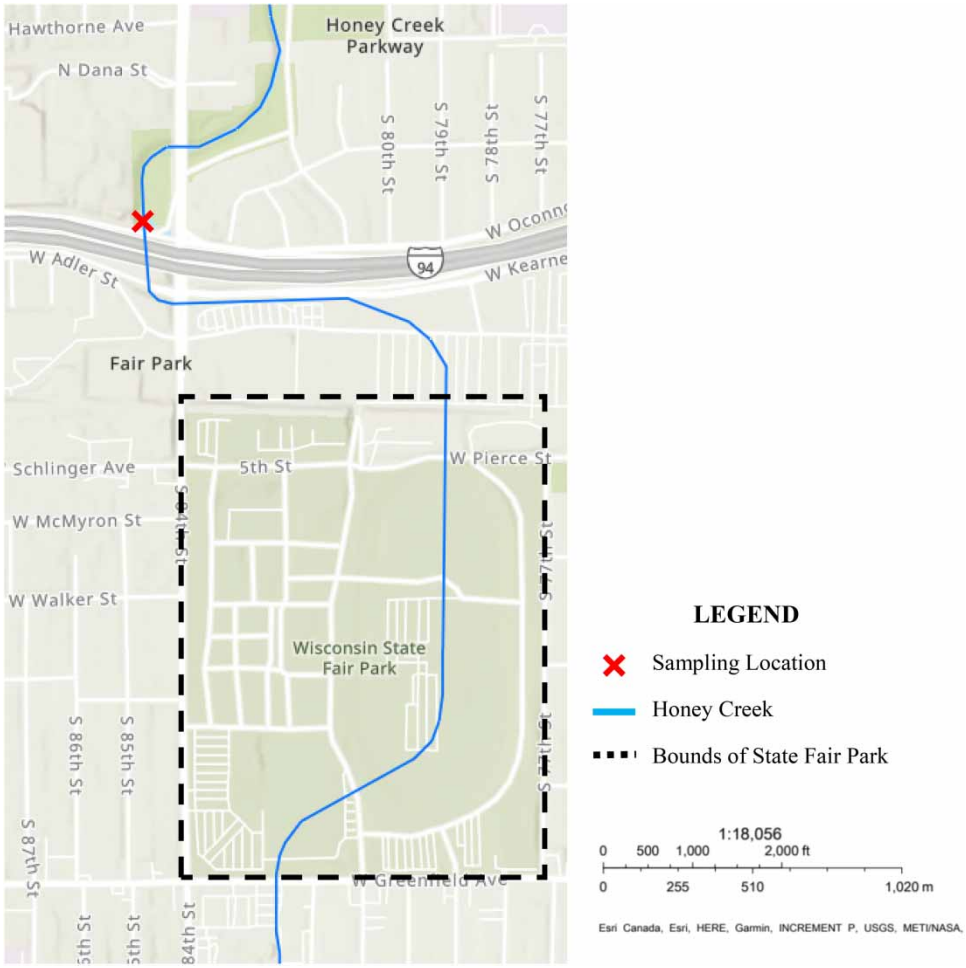

Figure 1 | Map of the sampling location in the Honey Creek, showing the boundaries of the Wisconsin State Fair Park with the continuation of the creek in a conduit underneath the fairgrounds. The map was generated with ArcGIS Online by Esri.

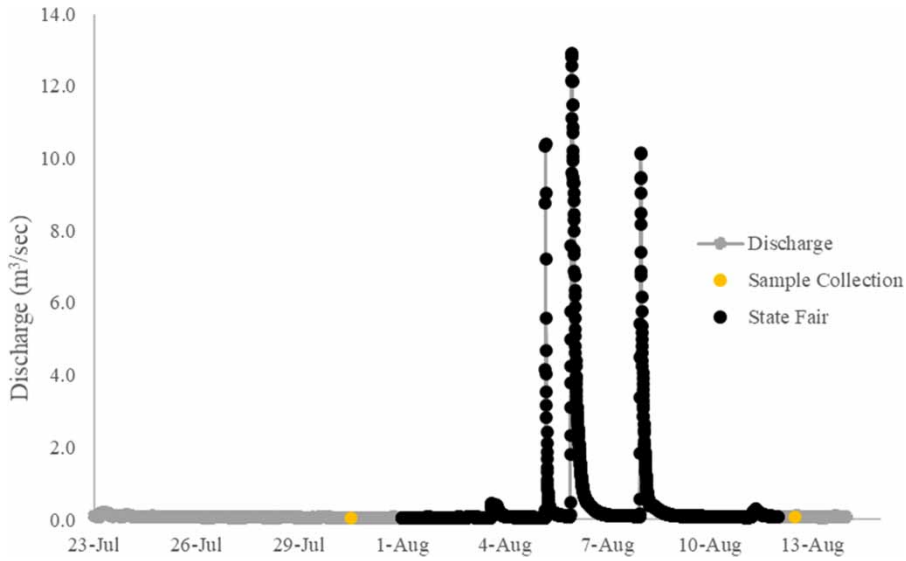

Figure 2 | 5-min interval discharge recorded at Honey Creek USGS stream gage in m^3/s from July 23rd to August 13th, 2019 (gray); samples in the creek were taken on two separate days, July 30th and August 12th (yellow); the Wisconsin State Fair ran between August 1st and 11th, 2019 (black).

discharge was less significant than the large storm events, the in-stream flow was still approximately five-times that of the average baseflow. In addition, there was no rain for 10 days prior to the first sampling event.

DNA extraction

After being transported to the lab, the 1-L water samples were filtered through a vacuum filtration apparatus using a 0.22 μm Merck Millipore Express Plus® membrane filter. Filters were subsequently cut into fragments and DNA was extracted from the filters according to the FastDNA™ Spin Kit manufacturer's protocol with minor modifications (MP Biomedicals, Santa Ana, CA). 1.0 mL of cell lysing solution for tissues and cells (CLS-TC) was used and in modification to the specified protocol, cells were lysed using liquid nitrogen freeze–thaw cycling (3×) rather than homogenization (Kappell *et al.* 2019). DNA extracts were stored at −20 °C until further analysis.

Gene quantification

For the three samples taken before and after the Wisconsin State Fair, three technical replicates of the ARG, bla_{TEM}, the integrase gene of the class 1 integrons, *intI1*, and the 16S rRNA gene were quantified ($n = 3$) via ddPCR. ddPCR assays were 22 μL reaction mixtures consisting of 11 μL QX200 ddPCR EvaGreen Supermix (Bio-Rad Laboratories Inc., Hercules, CA), 0.55 μL forward and reverse primers (250 nM each) (Table B2), 4 μL diluted (1:100) DNA extracts, and 5.9 μL Sigma® Life Science Molecular Biology Reagent Water. Following, 20 μL of the reaction mixture was pipetted into a 96-well plate, sealed, vortexed, and centrifuged to ensure homogenization. Droplets were generated in the QX200 Droplet Generator using an eight-channel cartridge (Bio-Rad). 20 μL of each reaction mixture and 70 μL of QX200 Droplet Generation Oil for EvaGreen (Bio-Rad) were dispersed into the respective, separate wells. 40 μL of the generated droplets were pipetted into a new 96-well PCR plate and sealed using a PX1 PCR Plate Sealer at 180 °C (Bio-Rad). Thermal cycling followed under the conditions of 5 min at 95 °C for activation of DNA polymerase, 39 cycles at 95 °C for 30 s and 60 °C for 60 s, followed by signal stabilization at 4 °C for 5 min and 90 °C for 5 min. The targeted genes were quantified using the QX200 Droplet Reader (Bio-Rad). Quality assurance and quality control according to the dMIQE checklist method were completed and documented to validate the ddPCR method (Supplementary Table B1; Huggett *et al.* 2013). Moreover, the limit of blanks, detection, and quantification were quantified according to the MIQE guidelines (Bustin *et al.* 2009; Deprez *et al.* 2016; Taylor *et al.* 2017).

Statistical analysis

The results of the ddPCR process were analyzed using by QuantaSoft™ version 1.7.4.0917 software. The software counts the positive and negative reactions of the generated droplets. Then, using the positive and negative relationship and the Poisson statistics, the concentration of the target DNA template of the sample was quantified. If a low number of droplets were

measured (<10,000 per 20 μL PCR), the reaction was rejected (Košir *et al.* 2017); the average number of accepted droplets was 14,500. A Student's *t*-test was performed using GraphPad Prism 7® (GraphPad Software, La Jolla, CA) to assess the difference between the gene abundances of the sampling events for all genes quantified ($\alpha \leq 0.05$).

RESULTS AND DISCUSSION

The 16S rRNA gene was quantified as a marker for total biomass in the stream before and after the state fair. Statistical analysis indicated a significant increase in the absolute abundance of the 16S rRNA gene after the fair when compared to before ($p<0.05$) (Supplementary Fig. A1). This result is in line with a previous survey of the stream, where bacterial loads in Honey Creek, evaluated through fecal coliform and *E. coli* analyses, displayed a distinct increase following the State Fair (Milwaukee Metropolitan Sewerage District 2006). The increase in the bacterial levels in this case was attributed to stormwater runoff, storm sewer discharges, and leaky sanitary sewer contamination. Previous research, moreover, has documented the impact of agricultural fecal contamination on surface waters, noting a persistence of fecal bacteria for weeks to months in the environment (Chee-Sanford *et al.* 2009; Joy *et al.* 2013).

The absolute abundance of the class 1 integron–integrase gene *intI1* after the Wisconsin State Fair was significantly higher than before ($p<0.001$) (Figure 3). Notably, the relative abundance of *intI1* (gene copies normalized to 16S rRNA) in the stream also exhibited a statistically significant increase between the sampling events (Student's *t*-test, *p*-value<0.01; Supplementary Fig. A2). The distinct rise of *intI1*, a gene known to promote horizontal gene transfer, following the Wisconsin State Fair, independent of a rise in the 16S rRNA gene, pinpoints the runoff from the fairgrounds as a source of *intI1* entering the urban water cycle.

The *intI1* gene, specifically, displayed a 1.14-fold increase in the logarithmic mean of the absolute gene copy concentration between the sampling events. *intI1*, a mobile genetic element, is often associated with ARG dissemination due to its enablement of horizontal gene transfer and its ability to accumulate and express a broad range of gene cassettes, including ARGs (Gillings *et al.* 2015). While *intI1* does not confer resistance directly, it was quantified in this case due to its ability to acquire exogenous ARGs and facilitate their transmission to pathogenic and nonpathogenic bacterium (Gillings 2014; Amos *et al.* 2018). Integron gene cassettes have been found to carry genes that confer resistance to most antibiotic classes, with *intI1* exhibiting a significant co-occurrence with sulfonamide and tetracycline resistant genes (Cheng *et al.* 2013; Gillings 2014). The presence of *intI1* in the environment, thus, is vast with approximately 15% of genome-sequenced bacteria carrying the gene (Gillings 2014). Class 1 integrons, moreover, can occur at a higher frequency in livestock when antibiotics have been administered. Up to 80% of Enterobacteria isolated from farm animals have class 1 integrons present (Ebner *et al.* 2004; Marchant *et al.* 2012; Gillings *et al.* 2015). Consequently, the concentration of *intI1* is used frequently as a representative measurement of resistance, selective pressures, and anthropogenic pollution in the environment (Gillings *et al.* 2015). The observed loads of *intI1* in the stream samples collected in this study suggest that the runoff from the state fairgrounds could be a hotspot for ARG transfer mediated between bacterial species by integrons, with elevated concentrations after the state fair event a probable result of animal-based contamination.

While samples were taken before the Wisconsin State Fair and the day after it ended, no intermediate samples were taken during the event to characterize the storm event ARG concentrations. Even so, the elevated ARG concentrations in the creek reveal that ARG contamination can persist following a major event. This persistence is consistent with a previous monitoring study at the same location that found elevated bacteria loadings several days after the run of the State Fair (Milwaukee Metropolitan Sewerage District 2006). A further limitation of this study is that data from only two points in time and one location are being used to characterize the conditions of Honey Creek, thus limiting the understanding of the temporal and spatial dynamics of ARGs in the stream. Temporally, it may be challenging to draw firm conclusions about the background data based upon two points in time; however, the significant increase in the concentration of ARGs would indicate the introduction of a source between these two sampling periods. Spatially, there is an unlikely chance that other pollution events upstream and independent of the State Fair were unobserved and could have affected the ARG concentration quantified in the urban stream. Given these limitations, future research could aim to sample throughout the Honey Creek and State Fairground to elucidate the specific factors contributing and influencing ARGs temporal and spatial dynamics. In this study, five separate storms were recorded between sampling; however, this study did not include samples taken at the time of a storm. The findings of this study are, therefore, likely an underestimate of the peak ARG loading that occurred in the stream at the time the stormwater runoff and discharge was conveyed from the event grounds. In addition,

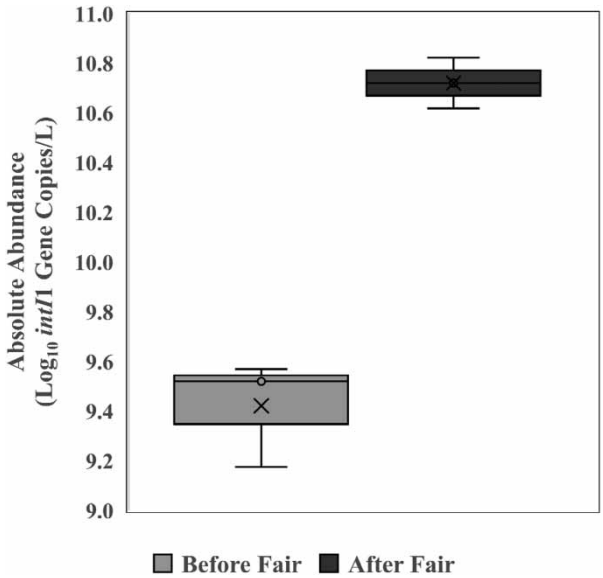

Figure 3 | Box plot displaying median distribution of the absolute gene abundance of class 1 integron–integrase *intI1* for samples taken before the Wisconsin State Fair ($n = 3$) and after ($n = 3$) as measured with ddPCR; a statistical difference was found between the absolute abundances of the sampling events (Student's *t*-test, *p*-value <0.001).

while this study cannot completely rule out other potential sources for the elevated ARGs, such as the mobilized sediment, both samples are taken during base conditions making mobilization of the sediment as a source unlikely. Given the likelihood of the increase being attributable to the state fair, there are several outcomes we can draw from the specific genes studied.

The ARG quantified in this study, bla_{TEM}, also exhibited a statistically significant increase ($p<0.05$) in the gene abundance after the Wisconsin State Fair (Figure 4). The relative abundance of bla_{TEM} after the fair, in contrast, was not significantly different than before (Student's *t*-test; Supplementary Fig. A3). The ARG bla_{TEM} confers resistance to β-lactams, a group of antibiotics (e.g. penicillin, cephalosporins, and carbapenems) widely prescribed for gram-negative bacterial infections (Zaniani *et al.* 2012). Beta-lactam resistance has been associated with the exacerbation of many serious infections and foodborne outbreaks originating from Enterobacteriaceae, specifically *Klebsiella pneumoniae* and *E. coli* (Zaniani *et al.* 2012; Bajpai *et al.* 2017). The CDC has labeled

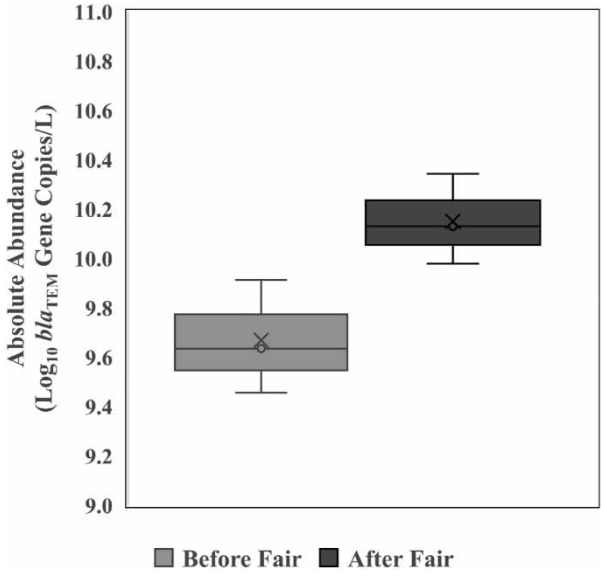

Figure 4 | Box plot displaying median distribution of the absolute gene abundance of bla_{TEM} for samples taken before the Wisconsin State Fair ($n = 3$) and after ($n = 3$) as measured with ddPCR; a statistical difference was found between the absolute abundances of the sampling events (Student's *t*-test, *p*-value <0.05).

β-lactamase-producing Enterobacteriaceae as a serious public health threat, with an estimated 197,400 hospitalized infections in 2017 attributable to $1.2 billion in healthcare costs (Center for Disease Control and Prevention 2020). Clinically, β-lactam resistance has been documented since the 1970s; in the aquatic environment, recent investigations have primarily focused on the identification of β-lactamase-resistant genes in wastewater discharge (Korzeniewska & Harnisz 2013; Coertze & Bezuidenhout 2019; Dunn *et al.* 2019). The sampling events presented in this study quantified the abundance of the bla_{TEM} gene in an urban stream, with the absolute logarithmic concentration of the bla_{TEM} gene increasing by 1.05-fold between samplings. The reservoirs of resistance created through runoff can activate the mobilization of ARGs between bacteria, including human pathogens (Martínez 2012; Subbiah *et al.* 2016). Consequently, stormwater has known routes of dissemination to humans through the contamination of drinking water sources, irrigation waters, and recreational waters (Nnadozie & Odume 2019). The contamination of the urban stream analyzed in this study is likely increasing the risk of exposure to humans and intensifying the already serious public health crisis of β-lactamase-resistant infections.

This study demonstrates the unique impact that short-term events like the Wisconsin State Fair can have on microbial water quality in urban streams. Moreover, short-term events specifically characterized by the increased presence of farm animals pose an additional threat toward ARG pollution as antibiotics such as tetracyclines, penicillin, and cephalosporins have long been used in animal production as nontherapeutic growth enhancers and to prevent disease (Subbiah *et al.* 2016). This use of antibiotics in animals can alter the gut resistome and result in the discharge of resistant bacteria, ARGs, and antibiotics in animal waste streams (Van Den Bogaard & Stobberingh 2000; Bengtsson-Palme 2017). Furthermore, there is evidence of animals who do not receive antibiotic treatments still acquiring ARGs due to mere proximity to human populations (Allen *et al.* 2010). ARGs associated with the use of penicillin, aminoglycosides, macrolides, tetracyclines, and sulfonamides have been found in the waste streams of animals (Landers *et al.* 2012; Tasho & Cho 2016). In contrast, stormwater and surface water sampling have revealed a different variety of ARGs and bacteria. Fosmidomycin and multi-drug resistance genes have been discovered in wet weather waters (Baral *et al.* 2018); a high prevalence of *intI1*, *sul1*, bla_{SHV}, *merA, strB*, and *qacF* have been observed in a storm drain outfall (Ahmed *et al.* 2018); and streptomycin-, gentamicin-, nalidixic acid-, vancomycin-, sulfamethoxazole-, and cefazolin-resistant isolates have been observed in surface waters impacted by stormwater (Zhang *et al.* 2016). The large-scale mixing of the indigenous microbial community of the receiving waters with the exogenous bacteria and genes from the stormwater with the possible addition of farm animal waste provides the ideal selection and ecological conditions for the emergence of novel resistance mechanisms and pathogenic strains (Tacão *et al.* 2012; Wellington *et al.* 2013; He *et al.* 2016). Consequently, inputs from short-term events, though occurring over a short period, are introducing a unique microbial community not normally discharged to the urban environment.

CONCLUSION

This study quantified the presence of genetic determinants in an urban stream on two separate occasions via ddPCR. The sampling quantified the impacts of a short-term event, the Wisconsin State Fair, can have on an urban stream. The average absolute abundance of *intI1* and bla_{TEM} between the two sampling events increased by 1.14- and 1.05-fold, respectively. The increase in loading demonstrates that state fairs can serve as a conduit of mobile genetic resistant elements and ARGs into the environment. Once in the environment, ARGs can generate advantageous phenotypes that will further shape the resistomes of the indigenous bacterial communities with adaptive responses, while mobile genetic elements can initiate horizontal gene transfer in bacteria (Gillings *et al.* 2009; Gillings 2014; Amos *et al.* 2018). The outcomes from this study support the need for further research that can produce a greater understanding of the magnitude in the rise of ARGs in relation to animal sources, runoff pathways, and treatment approaches during state fairs or other short-term events. Understanding this will help explain how short-term events that introduce unique microbial communities ultimately impact long-term microbial water quality of urban water cycles.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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