

# Antimicrobial resistance gene surveillance in the receiving waters of an upgraded wastewater treatment plant

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## Abstract

Wastewater treatment plants (WWTPs) have been identified as hotspots for antimicrobial resistance genes (ARGs) and thus represent a critical point where patterns in ARG abundances can be monitored prior to their release into the environment. The aim of the current study was to measure the impact of the release of the final treated effluent (FE) on the abundance of ARGs in the receiving water of a recently upgraded WWTP in the Canadian prairies. Sample nutrient content (phosphorous and nitrogen species) was measured as a proxy for WWTP functional performance, and quantitative PCR (qPCR) was used to measure the abundance of eight ARGs, the *intI1* gene associated with class I integrons, and the 16S rRNA gene. The genes *ermB*, *sul1*, *intI1*, *bla<sub>CTX-M</sub>*, *qnrS*, and *tetO* all had higher abundances downstream of the WWTP, consistent with the genes with highest abundance in the FE. These findings are consistent with the increasing evidence suggesting that human activity affects the abundances of ARGs in the environment. Although the degree of risk associated with releasing ARGs into the environment is still unclear, understanding the environmental dimension of this threat will help develop informed management policies to reduce the spread of antibiotic resistance and protect public health.

**Key words:** antimicrobial resistance gene, gene quantification, wastewater treatment, mobile genetic elements, extended-spectrum  $\beta$  lactamases

## OPEN ACCESS

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## Introduction

Although antimicrobial resistance genes (ARGs) can be detected in pristine environments with minimal anthropogenic influence, their measured abundance can be elevated in managed environments such as livestock feedlots and wastewater treatment plants (WWTPs). ARG abundances in these environments can be upwards of two orders of magnitude greater than those from natural environments (Li et al. 2015). WWTPs receive feces from hospitals and all residential households within the catchment area and therefore represent a critical control point where patterns in ARG abundances can be monitored prior to their release into the environment.

WWTP design is mainly focused on the effective removal of nutrients and enteric pathogens. Although these reductions are undoubtedly essential for reducing detrimental impacts to aquatic ecosystems caused by, for example, nitrogen pollution (Donald et al. 2011; Waiser et al. 2011), the

treatment process will invariably exert different selective pressures on bacterial communities, which can alter the effluent community diversity and species composition as well as the antibiotic resistance genes they harbour.

The resistome of wastewater influent, sludge, and effluent has been explored extensively. However, the effluent-receiving waters downstream of WWTPs have been less of a focus of previous literature. Several studies have taken a simplified approach to the downstream impacts of WWTP effluent, sampling only one site upstream and one downstream (Marti et al. 2013; Makowska et al. 2016), whereas others have explored complicated watershed systems with numerous ARG inputs (Devarajan et al. 2016; Xu et al. 2016). The WWTP of focus in this study is located in Regina, Saskatchewan, Canada, and recently underwent a \$175 million CAD upgrade to incorporate advanced biological nutrient removal processes to accommodate recent changes in governmental requirements for reduced nutrient release in the receiving waters of wastewater effluent. The potential impact of this WWTP on the environmental dimension of antibiotic resistance is unique due to the small size of receiving water of Wascana Creek whereby the WWTP effluent contributes to much of the downstream flow. Although sediment has frequently been the sample of choice in similar studies (Pruden et al. 2006; Marti et al. 2013), due to its implications as a reservoir of ARGs, we opted to sample surface water because of the inherent risk associated with the use of water containing ARGs for recreational or irrigation purposes (Ashbolt et al. 2013). Our findings are consistent with the increasing pool of evidence suggesting that human activity greatly affects the abundances of ARGs in the environment, but they also identify patterns in ARG abundance different from those of other regional studies, suggesting that unique mechanisms may influence ARG communities in different regions.

## Methods

### Site description

This study took place within the region of Wascana Creek that is adjacent to the Regina Municipal Wastewater Treatment Facility in southern Saskatchewan, Canada. The Wascana Creek watershed covers an area of approximately 3 870 km<sup>2</sup>, and contains over 20% of the province's population (Miki 2015). The creek originates near Vibank, Saskatchewan, flows through Regina, and ends west of Lumsden, Saskatchewan, where it joins the Qu'Appelle River. Approximately 7 km downstream of the Regina city boundary, the creek receives an average of 70 mL/d of wastewater, including hospital effluent and storm water. The flow rate and volume of Wascana Creek is low relative to the effluent-receiving rivers of other major Canadian cities and the effluent discharge represents >90% of the downstream flow for much of the year. The Regina WWTP was built in 1960 and was extensively upgraded during 2014–2016 to accommodate new effluent standards put into place by federal and provincial governments in Canada (Wastewater Systems Effluent Regulations SOR/2012-139). Upgrades included the installation of bioreactors to remove phosphorus and nitrogen, and improved UV disinfection of the final treated effluent (FE). Although the WWTP was under construction during the three months of sampling, the nutrient removal process remained relatively consistent, using a combination of the newly installed bioreactors and components of the old plant. Alum and polymers, which are used to aid in phosphorus sequestration and small particle flocculation, were used in treatment at the WWTP throughout the sampling dates.

### Sample collection

Surface water samples were collected in triplicate from seven sites: three upstream sites, abbreviated U1, U2, U3, and four downstream sites, abbreviated D0, D1, D2, D3 (Fig. 1). Sites U3, U2, and D3 were collected from a bridge, while D2, D1, D0, and U1 were collected 2 m from the edge of the shore. Given the small width of Wascana creek (<4.5 m), we estimate the difference between sampling



**Fig. 1.** Sampling overview of Wascana Creek surrounding the Regina wastewater treatment plant (WWTP). Sites upstream of the WWTP are designated with the letter U, while downstream sites are designated with the letter D. Image taken from Google Maps, © 2015 Google Inc., and used with permission.

approaches to be negligible. Additionally, samples of the FE were collected prior to its release from the plant. Samples were collected during six sampling events on 6 and 19 July, 11 and 22 August, and 6 and 12 September 2016. Sampling trips were conducted no fewer than 2 d after a heavy rainfall event (>15 mm of precipitation) to minimize the effect of flow rate on results. Detailed site information can be found in [Table S1](#).

## Sample processing

Samples were transported and stored at 4 °C for no longer than 48 h prior to processing. Between 100 and 150 mL of each sample was passed through a 47 mm diameter, 0.45 µm pore size filter (EMD Millipore, Billerica, Massachusetts, USA), in duplicate via vacuum filtration. The two filters were aseptically folded and retained in two sterile microfuge tubes and stored at –20 °C for later DNA extraction. The resulting filtrate was used for nutrient analysis, including total nitrogen, total phosphorus (TP), ammonia (NH<sub>3</sub>), soluble reactive phosphorus, nitrate (NO<sub>3</sub>), and nitrite (NO<sub>2</sub>). Nutrient analysis was completed at the Institute of Environmental Change and Society (University of Regina, Regina, Saskatchewan, Canada) using established protocols (Lachat Instruments, Loveland, Colorado, USA). For DNA extraction, each filter was briefly macerated with a sterilized metal spatula prior to cell lysis and homogenization, which was completed in the FastPrep®-24 Instrument (MP Biomedicals, Santa Ana, California, USA) at 4.0 m/s for three 1 min intervals. Subsequent DNA extractions were performed using the Powersoil® DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, California, USA) according to the manufacturer's instructions. DNA was pooled for the two replicate filters of each sample.

## Quantitative PCR of specific ARGs

The ARGs targeted in this study encode resistance to commonly prescribed antibiotic classes, such as β-lactams, macrolides, tetracyclines, sulfonamides, and fluoroquinolones (*bla*<sub>CTX-M</sub>, *ermB*, *tetO*, *sulI*, *qnrS*, respectively). The resistance genes associated with frequent hospital outbreaks of methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE), *mecA* and

*vanA*, respectively, were also targeted in this study. Additionally, we quantified the abundance of the class I integron integrase gene *intI1*, which has been correlated with anthropogenic inputs of ARGs within the environment (Gillings et al. 2015). Quantification of 16S rRNA gene amplicons (Suzuki et al. 2000) was used to normalize the ARG concentrations across sampling locations.

Quantitative PCR (qPCR) assays included the use of Taqman Environmental Mastermix 2.0 (Applied Biosystems, Foster City, California, USA) according to the manufacturer's specifications and were completed on a StepOnePlus™ Real-Time PCR System instrument (Applied BioSystems) using default settings for standard curve experiments. Each qPCR assay was done in duplicate and quantified according to a five-point standard curve over five orders of magnitude. For the scope of this study, values were considered below the limit of detection (LOD) if the qPCR assay reported no amplification, and below the limit of quantification (LOQ) if the reaction cycle threshold value was above that of the lowest point on the associated standard curve. Standards were created by cloning PCR amplicons of each gene of interest into a pGEM Easy-T cloning vector (Promega, Madison, Wisconsin, USA). Primer and probe sequences and their associated average qPCR efficiencies,  $R^2$  values, and LODs are listed in Table S2.

## Statistical analysis

Gene copy numbers were calculated in the StepOnePlus software v2.3 according to the standard curve included in each qPCR run. Technical reaction replicates were averaged and exported for analysis. All data visualization and analysis was completed in R 3.3.2 (R Core Team 2016) using packages ggplot2 and viridis (Wickham 2009; Garnier et al. 2017). All statistical analysis was completed using gene copy number normalized to the 16S rRNA gene copy number and log transformed. Differences in gene abundances between sites were evaluated via ANOVA and the Tukey's honestly significant difference test using a critical value of  $\alpha = 0.05$  to assess statistical significance.

## Results

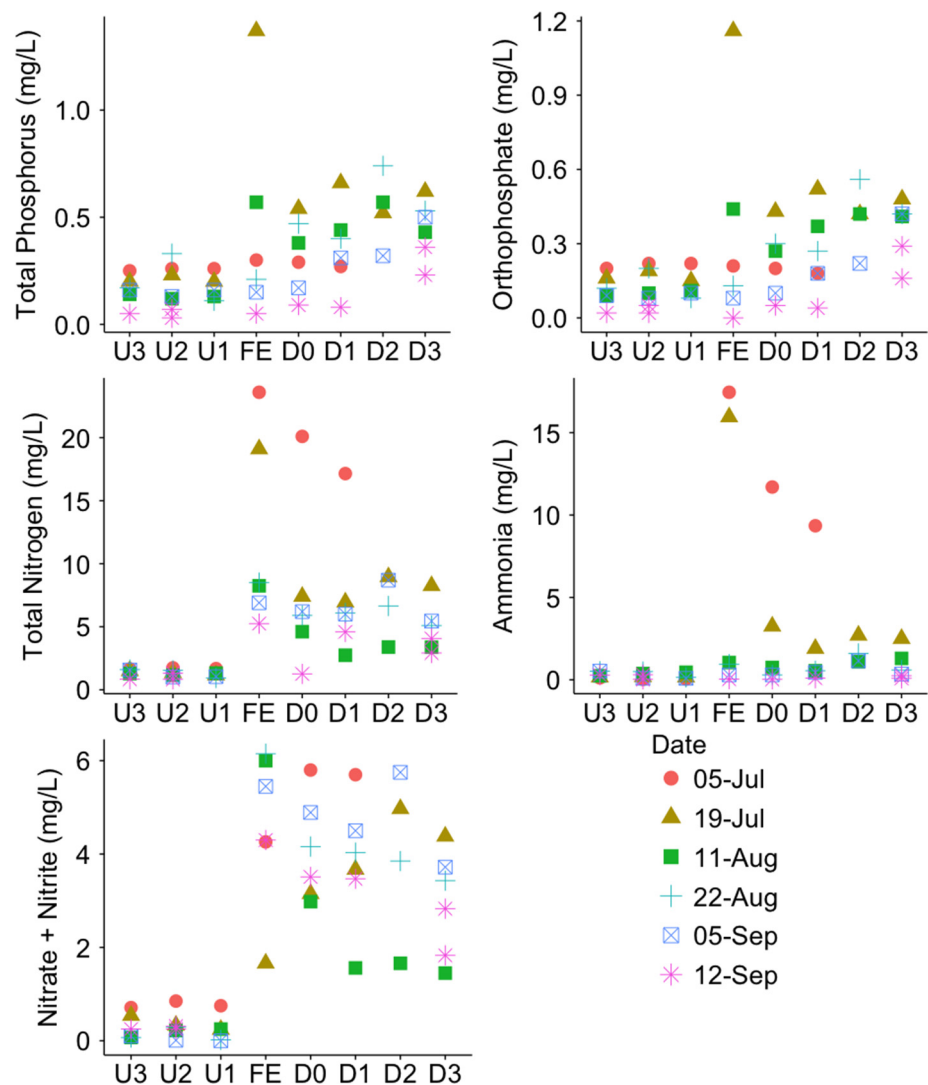
### Nutrient analysis and WWTP performance

According to the discharge permit, the TP content in the WWTP effluent was not to exceed 1 mg/L as determined by a six-month average of the daily tests. During the sampling period, TP content only once exceeded the permit limit on the 19 July sampling date, while the average across all dates was  $0.44 \pm 0.49$  mg/L (Fig. 2). A clear spike in nitrogen species can be observed in the effluent and downstream sites as compared with the upstream sites, indicating an impact of the release of the treated effluent on the nitrogen species content in the creek. Limits on the release of nitrogen species were not put into effect until January 2017. Furthermore, although spikes in nitrogen and phosphorus species can be observed in the effluent and downstream sites for both July sampling dates, concentrations remained consistently low in August and September, indicating that the WWTP performance improved during the final commissioning phase of the upgrades.

### Abundances of ARGs in the FE and upstream and downstream surface water samples

The 16S rRNA gene copy numbers were not found to differ significantly ( $p > 0.05$ ) between any of the sampling sites (Fig. 3). Therefore, prior to analysis, all ARG gene copy number values were normalized to their respective copies of the 16S rRNA gene. qPCR reaction efficiencies varied by primer set from 95% to 102% and all had  $R^2$  values  $>0.98$  (Table S2).

The genes with the highest measured abundances in the FE were *sul1*, *ermB*, and *intI1*, which had mean absolute copy numbers of  $3.1 \times 10^4$ ,  $2.5 \times 10^4$ , and  $8.1 \times 10^3$  per nanogram of DNA, respectively

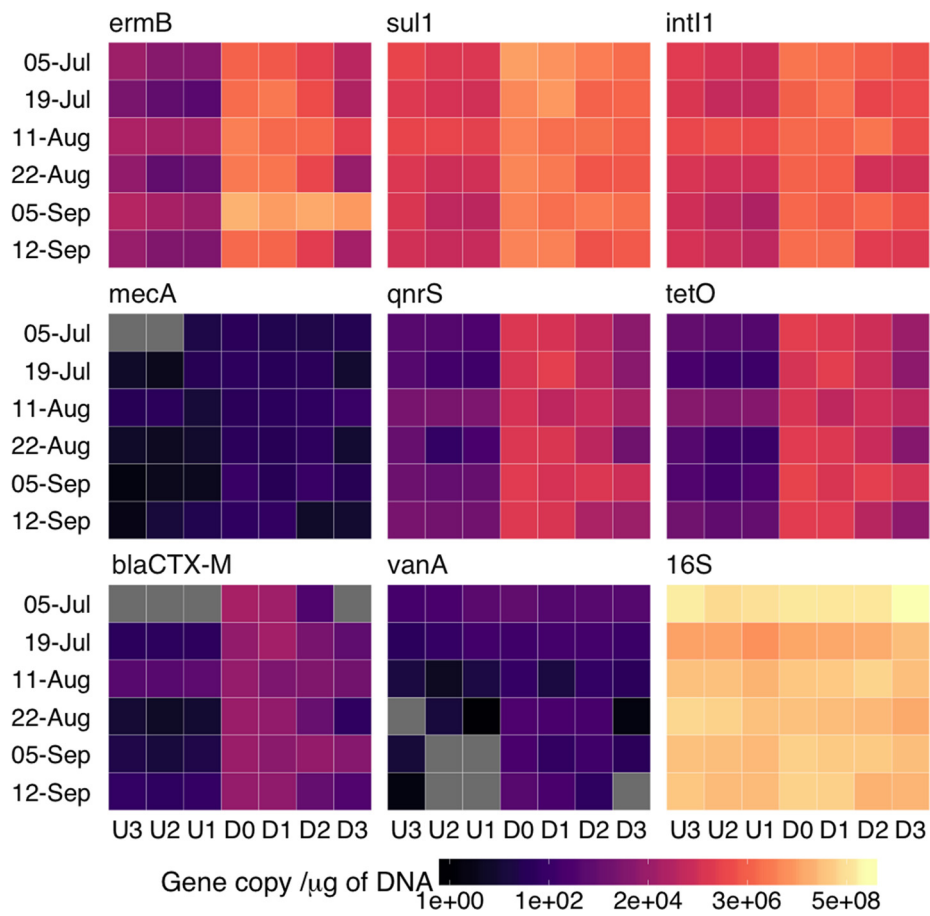


**Fig. 2.** Nitrogen and phosphorus species abundance across all sampled sites. With respect to the wastewater treatment effluent release point, U3 represents the farthest upstream site, D3 represents the farthest downstream site, and FE is the final treated effluent.

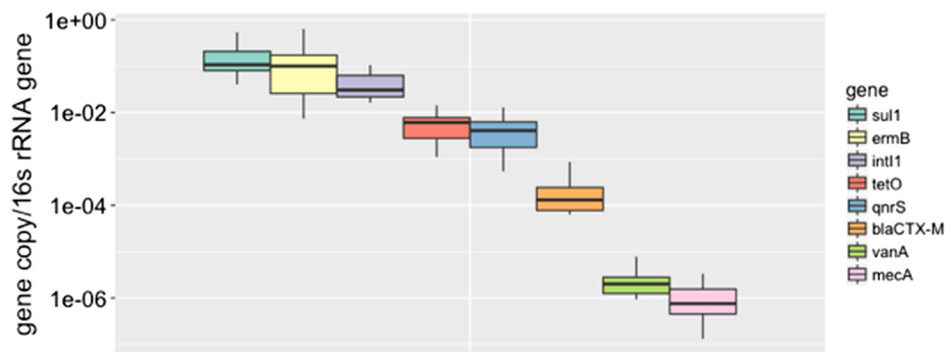
(Fig. 4). The plant released high levels of all measured genes relative to the surrounding environment (i.e., upstream samples, Figs. 3, 5), with the exception of *mecA* and *vanA*, which were occasionally below the LOD and consistently below the LOQ for all collected samples.

The three sampling points upstream of the plant were used to assess the ARG load of Wascana Creek prior to inputs from the plant. The abundance for each ARG gene was consistent and no significant differences ( $p > 0.05$ ) were observed between the three sites. The *vanA*, *mecA*, and *bla<sub>CTX-M</sub>* genes were present in upstream samples but below the LOQ.

Differences in the abundances of detectable ARGs were assessed between all downstream sites. Six out of the eight genes surveyed were found in higher quantities immediately downstream of the plant,

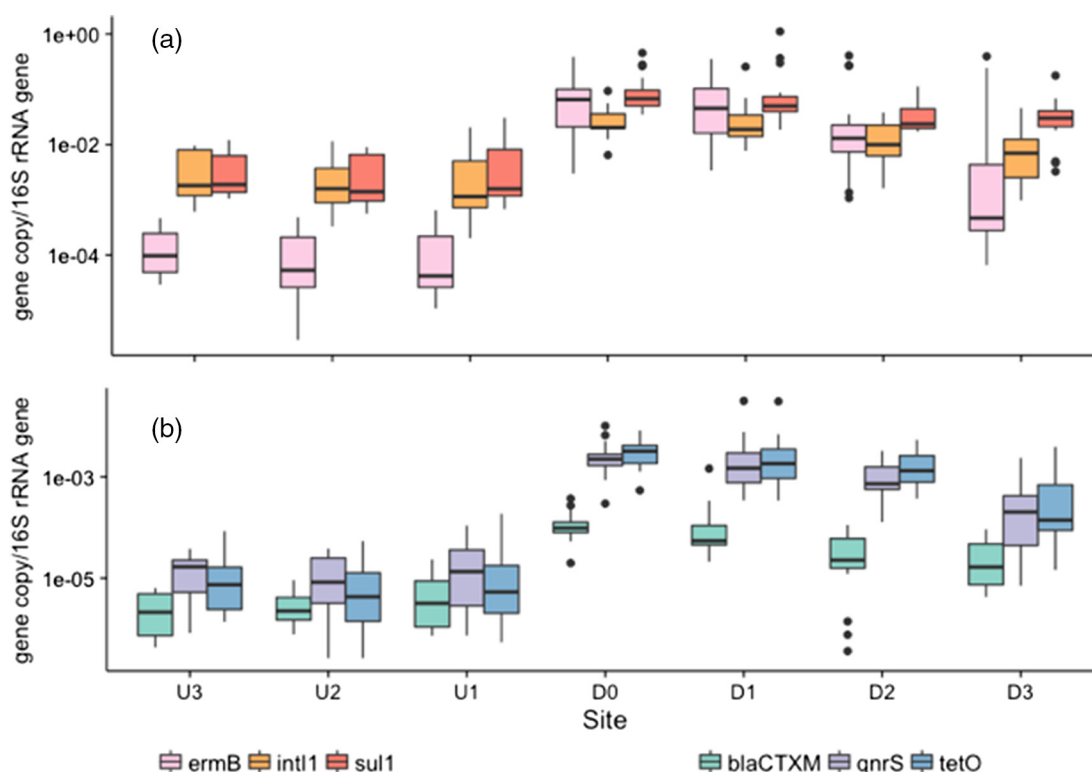


**Fig. 3.** Heat map of antimicrobial resistance gene abundance at each sampling site and for each date. Lighter colours indicate greater gene abundance, while darker colours indicate low abundance or absence. Sites U3 and D3 represent the farthest upstream and downstream sites from the plant, respectively. Grey squares represent samples where no amplification was observed.



**Fig. 4.** Mean gene copies per nanogram of DNA in the wastewater treatment plant final treated effluent. Each gene represents data pooled from three replicate grab samples across six sampling time points. The top, middle, and bottom of each box represent the 75th, 50th, and 25th percentiles, respectively. The vertical lines depict the range of the most extreme data points that are no more than 1.5× the length of the box away from the box; values outside this range are displayed with dots.





**Fig. 5.** Relative antimicrobial resistance gene abundances of selected genes through the creek sampling continuum. A range of relative abundances was observed, so for ease of visualization, they have been divided into (a) the top three most abundant genes and (b) the next three most abundant genes. The top, middle, and bottom of each box represent the 75th, 50th, and 25th percentiles, respectively. The vertical lines depict the range of the most extreme data points that are no more than 1.5 $\times$  the length of the box away from the box; values outside this range are displayed with dots.

including *ermB*, *sul1*, *int11*, *bla<sub>CTX-M</sub>*, *qnrS*, and *tetO* ( $p < 0.05$ ) (Figs. 3, 5). While the WWTP did increase the overall ARG abundance downstream of the plant, these changes were not uniform across all genes. *ermB* and *tetO* had the largest log increase in comparison with abundances upstream ( $2.62 \pm 0.46$  and  $2.59 \pm 0.67$ , respectively), while log increases in *int11* and *sul1* were considerably less ( $1.01 \pm 0.28$  and  $1.46 \pm 0.29$ , respectively).

The relative ARG abundances decreased in samples D1 through D3, corresponding with an increasing distance from the plant (Fig. 5). However, besides *ermB*, none of the genes surveyed returned to their upstream concentrations (U1–U3) by site D3, which is located approximately 5 km downstream of the effluent release point. Although the WWTP released high levels of *ermB*, its relative abundance decreased significantly by site D3 and thus did not differ from any of the upstream sites ( $p = 0.13$ ). The relative ARG abundances in water samples impacted by the wastewater effluent were still on average 1.03 log higher at site D3 compared with their respective U1 values (Fig. 5).

## Discussion

Although municipal wastewater can undergo extensive treatment for the reduction of biological nutrients and enteric pathogens, ARG-harboring bacteria can be released into the environment

(Cydzik-Kwiatkowska and Zielińska 2016). Similarly, our results indicate that the Regina Municipal WWTP is releasing treated effluent containing levels of ARGs that are higher in abundance relative to levels found in the surrounding environment.

Six of the eight measured genes were detectable upstream of the plant (*bla<sub>CTX-M</sub>*, *intI1*, *tetO*, *ermB*, *sulI*, and *qnrS*), which may not be surprising given that the creek flows directly through a mixed land-use area that includes agricultural lands and urbanized satellite communities with limited wastewater treatment. The creek contains quantified ARG levels that are consistent with studies focused on other similar urban rivers and creeks (Xu et al. 2016), which are far above expected ARG values for pristine aquatic environments (Marti et al. 2013).

Among the most abundant genes were the class I integron integrase gene *intI1* and *sulI* (Fig. 5). Their high abundance is unsurprising given that both have been identified as genetic markers associated with urbanization (Nardelli et al. 2012; Gillings et al. 2015). They are also remarkably similar in abundance, which is especially apparent in the upstream samples (Fig. 5). Their similar abundance may be attributed to the frequent co-occurrence of *sulI* with the conserved region at the 3' end of class 1 integrons (Bryskier 2005).

The treated effluent leaving the wastewater plant had increased abundances of most genes, with the noticeable exception of *vanA* and *mecA*. *vanA* and *mecA* were below the LOQ in the treated effluent, and at all creek sites. Although both genes are linked to notable outbreaks of resistant organisms (e.g., MRSA and VRE), they may be less likely to propagate within the WWTP environment. *vanA* encodes high level resistance to vancomycin (minimal inhibitory concentration (MIC): 1024 µg/mL) and teicoplanin (MIC: 512 µg/mL) and has been linked to numerous nosocomial VRE outbreaks (Abele-Horn et al. 2006). While *vanA*-associated vancomycin resistance is clinically widespread, the low frequency within wastewater-impacted environments maybe related to the genomic context whereby *vanA* is localized to a subset of *Enterococcus* spp. (Simjee et al. 2002). *mecA* is similar in that it has been found only in *Staphylococcus* and a few other select genera (Kassem et al. 2008) and is typically chromosomally encoded. It is unclear to what extent these genes are found in unculturable environmental bacteria.

*bla<sub>CTX-M</sub>* encodes resistance to extended-spectrum beta lactamases and is of significant clinical concern due to patient morbidity and mortality in treating infections caused by pathogens carrying the *bla<sub>CTX-M</sub>* gene (Paterson and Bonomo 2005). Although the *bla<sub>CTX-M</sub>* gene family is expansive and includes hundreds of variants, the primers used in this study capture the diversity found within the *bla<sub>CTX-M-1</sub>* cluster (Colomer-Lluch et al. 2011), which is frequently associated with mobile transposon elements and is thus of interest in a system where horizontal gene transfer can be prominent (Cantón et al. 2012). The effluent from the Regina WWTP increased the abundance of the *bla<sub>CTX-M</sub>* gene in the downstream aquatic environment, a phenomenon that has been observed in several other studied sites including arctic Canada (Neudorf et al. 2017), Portugal (Tácio et al. 2012), India (Devarajan et al. 2016), and Switzerland (Devarajan et al. 2015).

Many studies have surveyed the surface water and sediments of wastewater effluent-receiving waters and found that, in general, ARG abundances are increased downstream of a WWTP. However, the region of study appears to have a heavy effect on the presence and abundance of specific ARGs. For example, we detected and quantified *qnrS* consistently both upstream and downstream of the WWTP, whereas a 2014 study in Sweden could not, despite having a very similar study site (Berglund et al. 2015). Conversely, a different Swedish study consistently detected and quantified *mecA*, contrary to what we observed in this study (Börjesson et al. 2009).



Although this approach is limited in scope to the selected gene targets, it is an efficient and straightforward approach to monitoring the release of ARGs in treated wastewater effluent prior to release into the environment. While other similar technologies such as qPCR arrays and metagenomics sequencing may allow for the detection of more ARGs overall, the genes targeted in this study are representative of the resistance gene pool and are clinically relevant. Moreover, the selected approach can detect shifts in ARG abundances, which if monitored temporally, can provide insights into changing resistance patterns within the community.

## Conclusion

During this study, we observed elevated levels of six ARGs in the final treated wastewater of a municipal WWTP, as well as in the effluent-receiving body of water. Comparable studies in other countries have reported similar findings, indicating that this is a global phenomenon. Although the degree of risk associated with releasing ARGs into the environment is still unclear, understanding the environmental dimension of this threat will help develop informed management policies to reduce the spread of antibiotic resistance and protect public health.

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## Author contributions

KDN, LTH, RCJ, and CKY conceived and designed the study. CNF, LS, and KDN performed the experiments/collected the data. CNF and CKY analyzed and interpreted the data. LTH, RCJ, and CKY contributed resources. CNF, LTH, and CKY drafted or revised the manuscript.

## Competing interests

The authors have declared that no competing interests exist.

## Data accessibility statement

All relevant data are within the paper and in the Supplementary Material.

## Supplementary material

The following Supplementary Material is available with the article through the journal website at doi:[10.1139/facets-2017-0085](https://doi.org/10.1139/facets-2017-0085).

Supplementary Material 1

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