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Sources of Antibiotic Resistance Genes in a Rural River System

Mandy M. McConnell[a], Lisbeth Truelstrup Hansen[b*1], Kara D. Neudorf[b], Jenny L. Hayward[a],

Rob C. Jamieson[a], Chris K. Yost[c], Anthony Tong[d].

[a] Department of Civil and Resources Engineering, Dalhousie University, 1360 Barrington Street, Halifax B3H 4R2, Canada
[b] Department of Process Engineering and Applied Science, Dalhousie University, 1360 Barrington Street, Halifax B3H 4R2, Canada
[c] Department of Biology, University of Regina, 3737 Wascana Parkway, Regina, SK S4S 0A2, Canada
[d] Department of Chemistry, Acadia University, 6 University Avenue, Wolfville, Nova Scotia Canada B4P 2R6

[*]Corresponding author at: Technical University of Denmark, Denmark.
E-mail address: litr@food.dtu.dk (L. Truelstrup Hansen).
[1] Present address: National Food Institute, Technical University of Denmark, DK-2800 Kongens Lyngby, Denmark.

List of Abbreviations Used

AB	Antibiotic
AL	Aerated Lagoon
AR	Antibiotic Resistance
ARB	Antibiotic Resistant Bacteria
ARG	Antibiotic Resistance Gene
$CBOD_5$	Five-Day Carbonaceous Biochemical Oxygen Demand
E. coli	*Escherichia coli*
gDNA	Genomic DNA
HGT	Horizontal Gene Transfer
LOD	Limit of Detection
LOQ	Limit of Quantification
MGE	Mobile Genetic Element
qPCR	Quantitative Polymerase Chain Reaction
TSS	Total Suspended Solids
WWTP	Wastewater Treatment Plant

Core Ideas

-Tertiary wastewater treatment contributed antibiotic resistance genes (ARG) to the river

-ARG levels decreased as proximity to anthropogenic influence decreased

-ARGs were observed at detectable levels even in undeveloped headwaters

-High flow conditions correlated to high ARG loading in the river

-Positive correlations were found between ARGs and fecal indicators

Abstract

The increasing prevalence of antibiotic resistance genes (ARGs) in the environment is problematic due to the risk of horizontal gene transfer and development of antibiotic resistant pathogenic bacteria. Using a suite of monitoring tools this study aimed to investigate the sources of ARGs in a rural river system in Nova Scotia, Canada. The monitoring program specifically focused on the relative contribution of ARGs from a single tertiary level wastewater treatment plant (WWTP) in comparison to contributions from the up-gradient rural, sparsely developed, watershed. The overall gene concentration significantly ($p<0.05$) increased downstream from the WWTP, which suggested that tertiary level treatment still contributes ARGs to the environment. As a general trend, ARG concentrations upstream were found to decrease as proximity to human-impacted areas decreased; however, many ARGs remained above detection limits in headwater river samples, which suggested their ubiquitous presence in this watershed in the absence of obvious pollution sources. Significant correlations with ARGs were found for *HF183* human fecal marker, *Escherichia coli,* and some antibiotics, which suggested that these markers may be useful for prediction and understanding of ARG levels and sources in rural rivers.

1 Introduction

Effluents from wastewater treatment plants (WWTPs) have recently been shown to contribute to increased levels of antibiotic resistance (AR) in receiving environments by increasing levels of antibiotic resistant bacteria (ARB), antibiotic resistance genes (ARGs), and AR selective factors, such as antibiotics (ABs) (Berglund et al., 2015; Makowska et al., 2016; Proia et al., 2016; Rodriguez-Mozaz et al., 2015). Although WWTPs have been shown to decrease levels of ARGs throughout treatment, even tertiary effluents have been shown to contain considerable levels of ARGs, which are subsequently discharged into the environment (Rodriguez-Mozaz et al., 2015). Antibiotic resistance genes may persist even after selective pressures, such as ABs are removed making them extremely difficult to eliminate (Salyers and Amábile-Cuevas, 1997). Antibiotic resistance genes are often present on mobile genetic elements (MGEs) such as plasmids, transposons, and integrons, which allows for their horizontal gene transfer (HGT) between bacteria (Davies and Davies, 2010). Therefore, it is important to limit sources which pollute the environment with AR determinants, as once present, they can persist and spread, which leads to increased risk of creation of AR human pathogens.

Past studies have examined the effects of WWTP discharges on ARG presence in receiving waters in urban/anthropogenically impacted areas (Berlung et al., 2015). Proximity to, and increased levels of urbanization is often associated with higher levels of AR in the environment (Ouyang et al., 2015). However, the levels in relatively undeveloped watersheds have not been well documented. Quantification of ARG concentrations and loads in rural and remote areas, which are less influenced by anthropogenic activities, would help characterize the role that an individual WWTP has on contribution of ARGs to the environment, and provide insight into ARG levels that already exist in the environment in rural regions. Knapp et al. (2012)

also showed that seasonal changes in streamflow can influence the distribution and levels of ARGs in receiving water environments, which highlighted the demand for ARG monitoring programs to adequately capture hydrologic variability.

In recognition of these research gaps, the objective of this study was to characterize the relative role of an individual tertiary WWTP on ARG presence within a rural watershed under variable hydrologic conditions. A monitoring program was designed to measure both the concentration (copies/mL) and load (copies/second) of a suite of ARGs throughout the catchment upstream of the WWTP discharge to quantify background levels of ARGs in a relatively undeveloped, rural watershed. Sampling occurred during wet and dry events to assess how hydrology influences ARG abundances and loading. A suite of additional water quality indicators (generic *Escherichia coli* and human specific *Bacteroidales* markers) were also assessed to identify potential indicators of ARG presence, and help confirm potential sources.

2 Materials and Methods

2.1 Sampling sites and sample collection

Wastewater samples were collected from a municipal tertiary WWTP located in Greenwood, Nova Scotia, Canada (44° 57’ 42” N, 64° 55’ 44” W) that services a population of 6500 people (Statistics Canada, 2011). The WWTP discharges approximately 1 MGD (3.8×10^6 L/day) into the Fales River, which is tributary to the Annapolis River. There are between 600-800 customer connections, which includes a Canadian Forces airbase which contains a small hospital unit. Treatment units include primary clarifiers, two aerated lagoons (ALs), two secondary clarifiers, sand filtration, and UV disinfection. The plant must adhere to a strict regulatory effluent discharge requirement of a maximum content of 5 mg/L for five-day carbonaceous biochemical oxygen demand ($CBOD_5$) and 5 mg/L for total suspended solids (TSS).

Sample collection that included influent (I) [after primary clarifiers], effluent (E) [after UV disinfection], downstream (D), and upstream 1 (UP1) water samples was performed on August 18/2015 (summer/dry weather event), February 23/2016 (winter/dry weather event), April 12/2016 (early spring/wet weather event), May 10/2016 (late spring/dry weather event), and August 2/2016 (summer/dry weather event). Samples labeled as a dry weather event were collected during baseflow periods, while samples were labeled as a wet weather event if samples were collected during a storm hydrograph event. For the sampling dates April 12/2016, May 10/2016, and August 2/2016, an additional six upstream water sites were sampled (UP2 to UP7). Figure 1 and Table 1 illustrate the locations of these collection sites and provide characteristics of their drainage areas. Upstream sample collection sites were chosen to represent points along both primary tributaries in closer proximity of (UP2, UP3, UP4, UP6), and further away (UP5, UP7) from urbanization and human-impacted areas. The watershed delineation, stream network, and land use mapping was created using ESRI ArcGIS ArcMap 10© software. A hydrologically correct 20 m Digitial Elevation Model was sourced from Government of Nova Scotia (2006), and the Canvec geospatial dataset (Government of Canada (2016a,b) was used to conduct the land-use characterization.

Samples were collected in pre-sterilized 1 L Nalgene collection bottles (Thermo Fisher Scientific). Water samples were kept on ice while being transported back to the laboratory at Dalhousie University in Halifax, NS and stored in the fridge at 4°C until analysis. All samples were processed within their respective sample holding times.

2.2 Analysis of the Abundance of Antibiotic Resistance Genes, Human Fecal Markers and 16S rRNA Gene Copies

2.2.1 Genomic extraction

Microorganisms in wastewater and river samples were concentrated (in quantities ranging from 50 (influent) to 500 mL (effluent and river samples)) by filtration through 0.45 μM membranes using a vacuum manifold (Millipore, Inc., Bedford, MA). Filters were stored at -20°C until DNA extraction. Genomic DNA was extracted from the entire filter using the MoBio Powersoil DNA extraction kit (VWR International, Ville Mont-Royal, QC, Canada) according to manufacturer's specifications and stored at -20°C. The concentration and purity of the DNA were evaluated by ultraviolet absorbance spectrophotometry at 260/280 nm and 260/230 nm (Implen NanoPhotometer™, Implen, München, Germany).

2.2.2 Quantitative real-time PCR

Assessment of gene targets was performed using quantitative real-time PCR (qPCR). Primer and probe sequences and cycling conditions for nine antibiotic resistance genes and one integrase gene (*intI*) used as a proxy to assess ARG cassettes and mobility potential (Norman et al., 2009; Toleman et al., 2006), were obtained from the literature and are detailed in Supporting Material (Table S1). The genetic targets assessed in this study include class 1 integrase (*intI*), class A β-lactamase ($bla_{CTX\text{-}M}$ and bla_{TEM}), erythromycin resistance gene (*ermB*), fluoroquinolone resistance gene (*qnrS*), sulphonamide resistance genes (*sul1* and *sul2*), tetracycline resistance gene (*tetO*), methicillin resistance gene (*mecA*), and vancomycin resistance gene (*vanA*). These ARGs were chosen to represent a variety of different AB classes and resistance mechanisms as well as clinically relevant genes (Szczepanowski et al., 2009; Volkmann et al., 2004). Human-specific *HF183 Bacteroidales* 16S rRNA genetic marker was also quantified to examine a potential correlation between human faecal pollution and ARG abundance (Sauer et al., 2011). The concentration of bacterial 16S rRNA was also determined by qPCR to allow for the calculation of the relative abundance of ARG relative to the 16S rRNA copy numbers (Neudorf et al., 2017). All targets were quantified in all water samples and were run in duplicates for samples, and triplicates for positive control standards and no template controls (NTCs). Control plasmids for *int1*, bla_{TEM}, *sul1*, and *sul2* were obtained from Dr. E. Topp (University of Western Ontario, London, ON, Canada) and described in Rahube et al. (2014). Control plasmids for $bla_{CTX\text{-}M}$, *ermB*, *qnrS*, *tetO*, *mecA*, and *vanA* are described in Neudorf et al. (2017). Concentrations of plasmid DNA were quantified using Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific). Standard curves were constructed for each assay using tenfold serial dilutions of positive controls in triplicate. Quality assurance for standard curves were performed according to recommendations from the Bio-Rad Real-Time PCR Applications Guide (Bio-Rad, 2013). Efficiencies ranged from 87.3% to 114.5% and R^2 values were >0.99 for all calibration curves. Limit of quantification (LOQ) (copies/reaction) were as follows: *int1*=14.4, *sul1*=11.7, *sul2*=9.6, *tetO*=69.0, *ermB*=13.8, $bla_{CTX\text{-}M}$=6.2, bla_{TEM}=243.0, *qnrS*=112.0, *mecA*=69.0, *vanA*=138.0, 16S rRNA=67000, *HF183*=3630, which are similar to values reported in the original papers. Limit of detection (LOD) was 5 copies/reaction (or 1 copy/mL for 500 mL sample volumes and 10 copies/mL for 50 mL sample volumes).

TaqMan qPCR on a Bio-Rad CFX96 Touch system (Bio-Rad, Hercules, CA, USA) was used in quantification of *int1* and ARGs. The following reaction mixture was used: 1 x SsoAdvanced^{TM} Universal Probes Supermix (Bio-Rad), 0.9 µM of each primer, 0.25 µM TaqMan probe, 2 µl template DNA, and 2 µl of sterile nuclease-free water (Thermo Fisher) to a final volume of 20 µl. Water samples and negative controls without template DNA reactions were run in duplicate, while standards (control plasmid) reactions were run in triplicate. Taqman probes were also used in quantification of the human faecal *HF183* marker. The following reaction mixture was used: 1 x SsoAdvanced^{TM} Universal Probes Supermix (Bio-Rad), 0.6 µM of each primer, 0.25 µM TaqMan probe, 2 µl template DNA, and 6.5 µl of sterile nuclease-free water (Thermo Fisher Scientific) to a final volume of 25 µl. SYBR Green qPCR was used to quantify universal 16S rRNA gene fragments. The following reaction mixture was used: 10 µl Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, United States), 0.4 µM primers, and 1 µl of template DNA, and 7.4 µl of sterile nuclease-free water (Thermo Fisher Scientific) to a final volume of 20 µl. All SYBR Green qPCRs were run in triplicate including negative controls without template DNA.

2.3 Assessment of Water Quality

All samples were tested for the following water quality parameters: total suspended solids (TSS), volatile suspended solids (VSS), ammonia, nitrate, and *Escherichia coli*. Wastewater samples were also tested for $CBOD_{5,}$ total nitrogen (TN), total phosphorous (TP), and chemical oxygen demand (COD). TSS and VSS were performed using Whatman^{TM} 934-AH 47 mm glass fiber filters (Thermo Fisher Scientific) according to APHA standard methods 2540 D (American Public Health Association, 1999). Ammonia and nitrate were measured using high performance ammonia and nitrate electrodes, respectively, as directed by the manufacturer (Thermo Fisher Scientific). The electrodes were attached to an Orion Star^{TM} series meter (Thermo Fisher Scientific). *E. coli* was measured using IDEXX Colilert®-18 and Quanti-Trays® (IDEXX Laboratories, Inc., Westbrook, ME, United States) according to the manufacturer's instructions in IDEXX Laboratories, Inc. (2012). Analysis of $CBOD_5$ was performed in duplicate according to the APHA standard method 5210B (American Public Health Association, 1999). TN was analyzed using Hach® TN Test 'N Tubes™ (0.5 to 25.0 mg/L N, Hach Company, Loveland, CO, United States), according to the manufacturer's procedure. TP was analyzed using Hach® TP Test 'N Tube™ ranging from 1.0 to 100.0 mg/L $PO4^{3-}$ (Hach Company), following the manufacturer's procedure. COD was analyzed using Hach® COD TNT plus Vial™ Test ranging from 20 - 1500 mg/L COD (Hach Company), following the manufacturer's procedure.

Water quality measurements for temperature (°C), conductivity (μS/cm), dissolved oxygen (DO (both % and mg/L)), pH, and oxidation reduction potential (ORP (mV)), were conducted on samples *in-situ* using a handheld YSI 600R or 600QS multi-parameter water quality sonde (YSI Inc., Yellow Springs, OH United States). River flow rates were determined using the velocity – area method (Dingman, 2002). Velocity and depth were measured using a Gurley Precision Instruments (Troy, NY, United States) 625DF2N digital pygmy meter.

2.4 Antibiotics Analysis

The samples were also tested for a range of 10 clinically relevant antibiotics consisting of amoxicillin, cefaclor, cefprozil, cefdinir, levofloxacin, ciprofloxacin, azithromycin, clindamycin, clarithomycin, and triclocarban. A vacuum filter apparatus equipped with 3 mL Chromabond® HR-X (200 mg sorbent) solid phase extraction (SPE) columns was used to extract the target antibiotics. These SPE columns were conditioned with 6 mL methanol and 6 ml of deionized (DI) water. The pH was measured from 100 mL samples and adjusted to pH 2.5 ± 0.5 with 1 M HCl. The samples were then pumped through the column at 5 mL/min under vacuum. Cartridges were washed with 3 mL of 10% (v/v) methanol in DI and dried for 5 min under vacuum.

Analytes were eluted using 3 mL of methanol two times for a total of 6 mL. Eluent was collected and reduced to 1 mL with gentle nitrogen blowdown at approximately 37 °C. Antibiotic analysis was performed using an Agilent 1200 HPLC coupled with an Agilent 6410 triple quadrupole mass spectrometer. Chromatographic separation was performed using a 25-cm Agilent Poroshell Eclipse C18 column with a 4.6-mm internal diameter and 2.7-µm particles. The flow rate of mobile phase (a mix of methanol and ultrapure water) was 0.5 mL/min. A solvent gradient was programed to start at 20% (v/v) methanol for 0.5 min, which increased to 100% by 20 min, and then held for another 5 mins with a maximum flow gradient of 100 mL/min^2. The column was kept at a constant temperature of 40 °C. Following separation, ionization was conducted with an electrospray ionization (ESI) source under 35-psi nebulizer pressure. Drying gas temperature was set to 350 °C with a flow rate of 12 L/min. The MS was operated in the positive mode and the capillary voltage was held at 4000 V. Nebulizing collision gases were 98% nitrogen and ultra-high purity (UHP, 99.999 %) nitrogen., respectively. Precursor-to-product ion transitions were established for all target antibiotics. Quality assurance and quality control included the use of continuing calibration verification, solvent blanks, spiked samples, internal standards, and duplicate samples (technical replicates, n=2).

2.5 Data Analysis

Raw fluorescence data from the Bio-Rad qPCR system were processed using the LinRegPCR program (v 11.4) (Ruijter et al., 2009). In order to account for differences in efficiencies between samples and standards, a one-point calibration (OPC) method for absolute quantification was used (Brankatschk et al., 2012). For statistical calculations, any values that fell between the LOD and LOQ were set to ½ of LOQ value. Any values that fell below the LOD were set to ½ of the LOD. For calculation of the relative abundance of genes, the gene copy (GC) number of each gene was normalized to the GC number of the 16S rRNA gene in each sample and log transformed (log(GC/16S rRNA GC)). For calculation of the absolute abundance of genes, gene copies were normalized to the water volume used for gDNA extractions to generate GC per mL of water.

One-way analysis of variance (ANOVA), Tukey HSD test, and t-tests were used to assess statistically significant differences ($p < 0.05$) among samples. Pearson correlation coefficients were used to assess significant ($p<0.05$) correlations between ARGs and water quality parameters. Analysis was performed using GraphPad Prism version 7.00 for Mac OS X (GraphPad Software, La Jolla California USA, www.graphpad.com), and Microsoft Excel, 2016. Abundances for each individual ARG were pooled and averaged for each sampling site for the statistical analyses.

3. Results and Discussion

3.1 Presence and abundance of ARGs in WWTP influent and effluent samples

The majority of ARGs were detected in both the influent and effluent samples from all sampling events (August 18, 2015, February 23, 2016 April 12, 2016, May 10, 2016, and August 2, 2016). Exceptions included *mecA,* which was below the LOD on August 18, 2015 (all samples) and on August 2, 2016 (effluent), and *vanA,* which was below the LOD in all samples and will not be included in further analysis. There was a decrease of 1.93 log units from the influent to the effluent that was quantified with the pooled ARG absolute averages from the five sampling events. This indicated that the WWTP decreased overall ARG absolute abundance significantly ($p<0.05$) throughout treatment. The effluent leaving the WWTP consistently met the regulatory target values for both TSS and $CBOD_5$ (both 5 mg/L) during the sampling period, which indicated that the plant operated efficiently (Table S2). A decrease of ARGs throughout the treatment continuum is consistent with previous studies (Chen and Zhang, 2013; Gao et al., 2015; Laht et al., 2014; Munir et al., 2011). However, the effluent still contained an average of close to 3 log units of ARGs/mL even after tertiary level treatment (see Figure 2a). Therefore, although the effluent was reduced in ARG concentrations compared to influent, ARGs were still present at detectable concentrations.

3.2 Abundance and load of ARGs in water samples collected upstream (UP1) and downstream (D) of the WWTP effluent discharge point

Analyses of downstream (D) and upstream 1 (UP1) samples from August 2015, and February, April, May, and August 2016 sampling events revealed generally higher abundances of ARGs downstream from the WWTP than upstream (Figure 2a). All ARGs were detected in both UP1 and D samples, except for bla_{TEM} (<LOD in UP1 and D samples in May), $bla_{CTX\text{-}M}$ (<LOD in UP1 and D samples in April, as well as UP1 sample in May), *sul2* (<LOD in D sample in April), *mecA* (<LOD in both UP1 and D samples in August 2015 and August 2016 UP1 sample).

Although D samples often harboured a higher abundance of genes than UP1 samples, comparisons (t-test) of UP1 and D samples averaged from all sampling events for each ARG, showed only *ermB* was significantly ($p<0.05$) different, for both absolute (copies/mL) and relative abundance (copies/16S rRNA gene copies). These results may suggest *ermB* to be more tightly linked to anthropogenic impacted ecosystems, a finding which agrees with what was recently reported for river recipient in Saskatchewan, Canada (Freeman et al., 2017). Significant differences ($p<0.05$) were observed in absolute and relative abundances in the pooling the genes (8 ARGs and *int1*) for the UP1 and D samples. These results indicated that the WWTP contributed to increased overall ARG concentrations and to an increased proportion of bacteria which carried ARGs in water downstream from the plant.

The impact of the WWTP on the ARG concentrations in the river was higher during the August sampling event, as there were larger differences between upstream and downstream samples for most ARGs as well as higher concentrations than other months (Figure 2a). However, ARG loading (log copies/sec) measurements suggest that less total ARGs were transported in August (Figure 2b). River flow rates measured at the effluent discharge location varied from 0.06 m^3/s in August 2016 to 3.37 m^3/s in April 2016. Higher downstream concentrations in dry periods were caused by lower river levels and flows which caused the effluent discharge to contribute more to the water levels downstream; as well as there being lower upstream levels of ARGs during these sampling events. A study by Knapp et al. (2012) which examined a river in Cuba during both wet and dry seasons also found that waste outfalls more strongly influenced ARG levels in local downstream river samples in a drier season.

3.3 Concentration and loading of ARGs in the upstream watershed

The concentration and load of the ARGs in the upstream river samples displayed seasonal/weather event differences. For instance, April samples contained significantly ($p<0.05$) higher ARG concentrations (log copies/mL) and load (copies/second) compared to May (second most) and August (least) water samples (Figure 2). The highest stream flow was in April and coincided with peaks in ARG levels, which suggested that hydrologic processes (surface runoff, sediment resuspension) were likely involved in mobilization of ARGs. Knapp et al. (2012) also found seasonal variations in ARG levels in a river in Cuba, finding an increased overall abundance of ARGs in the river in wet seasons when compared to dry seasons.

Higher concentrations and loads of ARGs in the upstream river were observed at most sites in April with obvious "hotspots" observed in UP2, UP4, and UP6. Sites UP2, UP4, and UP6 contain some residential areas and a small of amount of agriculture in their drainage areas, which may have contributed to these seasonal peaks in ARG abundance, as these factors have been suggested to increase ARG levels (Ouyang et al., 2015).

Excluding the wet weather event in April 2016, gene concentrations followed a general trend of lower concentrations the further upstream and away from urbanization/agriculture the samples were taken. However, concentrations seemed to remain at a consistent level even at the UP7 site, which does not possess any known pollution sources within its drainage area. This suggests ARGs are present and can persist in the environment without known selective pressures, which has been shown in previous studies (Nesme et al., 2014). The load of ARGs at UP7 was actually higher in May and August than at the UP6 sampling site, which is more influenced by anthropogenic activities. ARGs being transported through the river at a relatively unimpacted sites suggests there may be unknown pollution source points, and also reconfirms that resistance can be measured everywhere (Nesme et al., 2014). Although there are no known residences or agricultural activities upstream of UP7, this area is used by local populations for hunting, fishing, and camping and by the military, which may be contributing to the presence of ARGs at this sampling location. Previous studies also reported higher ARG levels in more urban effected areas when compared to more undeveloped environments (upstream); however low ARG levels were still observed in samples from undeveloped areas (Ouyang et al., 2015; Pruden et al., 2006; Stortebloom et al., 2010).

Findings from this study and previous studies suggest that anthropogenic activities do increase the levels of ARGs in the environment; however, ARGs also exist in watersheds with no obvious human inputs. Even sub-lethal concentrations of ABs can sustain or exert a selective force for AR in the environment, and once ARGs are established they can be replicated and spread among bacteria (Martinez, 2009). It has been suggested that ABs have been produced naturally for over 500 million years (Baltz, 2008). Antibiotics and ARGs have therefore existed in the environment before human AB therapeutic uses came into effect (D'Costa et al., 2011). Despite this natural presence, human activities (e.g., medical and agricultural uses of ABs) have most likely increased the prevalence and spread of AR (Allen et al., 2010). Antibiotic resistance has been shown to be able to spread throughout the environment through mechanisms such as urban and agricultural runoff, wind, as well as biological forces such as animals and humans (Allen et al., 2010). It is also possible that genes that confer resistance to antibiotics in pathogens may have an alternative purpose in their original host, which may explain their presence in different compartments of the environment without typical selective pressures (Martinez, 2009).

The variations in individual ARG levels among the sampling sites in the watershed are shown for the ARGs in Figure 3. Most ARGs decreased as the samples were collected further away from urbanization. However, the detection of *mecA* was unexpected including its highest level of 3 log (GC/mL) in April and presence in some May upstream samples leading to the sequencing of amplicons from some samples to positively confirm gene identity. *MecA* confers resistance to antibiotics used as a last resort in clinical practices; therefore presence of this resistance gene was expected to be low in the environment due to lower clinical use. *MecA* is also often present on chromosomal DNA and therefore only carried by certain bacteria (e.g. *Staphylococcus aureus*), which may limit its environmental presence (Katayama et al., 2000). It is possible that during these higher flow conditions *mecA* entered the river through releases from melting snow, septic system discharges, storm runoff, presence of selective factors, and re-entrainment from sediment during the increased flow. *Int1*, *sul2*, *ermB*, *sul1*, $bla_{CTX\text{-}M}$ and *mecA* were the genes that seemed to be most affected by the wet weather sampling event in April. Likewise, *Sul2*, *ermB*, *sul1*, $bla_{CTX\text{-}M}$, and *mecA* were also not consistently present in upstream samples, which indicated that the genes which flushed into the river in high abundances in April seemed to be less commonly found in the river during other seasons. *Int1* and *tetO* remained present in consistently higher levels in water samples from both the river and WWTP compared to other ARGs. *TetO* confers resistance to tetracycline ABs, an older group of highly consumed ABs (Laht et al., 2014), which may explain its elevated presence.

3.4. Correlations between ARG abundance and fecal indicators

Figure 4 shows the concentrations of fecal indicator markers (*E. coli*, and *HF183* human *Bacteroidales* marker) in water samples obtained on the April, May and August sampling trips in 2016. Levels of *E. coli* decreased as a result of wastewater treatment and remained close to the detection limit in the more remote portions of the watershed (e.g., after upstream 3) for all seasons (Figure 4a). Significant ($p<0.05$) correlations were found between ARG concentrations and *E. coli* for all months (April $r=0.710$; May $r=0.820$; August $r=0.710$). The *HF183* marker—which detects faecal *Bacteroidales* of human origin and can be used as a proxy to measure human faecal pollution (Sauer et al., 2011) decreased throughout wastewater treatment following a similar log reduction as the ARG markers (Figure 4b and Figure 2a for *HF183* and total ARGs, respectively). *HF183* generally decreased the further the samples were taken upstream, however consistent presence were observed at the UP6 and UP7 sites (Figure 4b), which suggested an upstream source of human fecal contamination in the watershed, although *E. coli* levels were low in these samples. There were significant ($p<0.05$) correlations between *HF183* levels and ARG levels for each month (April $r=0.879$, May $r=0.845$, August $r=0.860$). *HF183* was the only parameter analyzed that seemed to "peak" in the April samples together with high ARG levels, which indicated that it may be useful in prediction of elevated ARG levels. It is possible that septic systems from nearby unmapped camps or outhouses as well as undocumented human use of the area, could be contributing to human fecal contamination and presence of ARGs in these areas.

3.5 Correlations between ARGs and antibiotics

The concentrations of ABs in water samples from April, May and August of 2016 are provided in Table S3. There were significant positive correlations in April ($r=0.805$, $p<0.05$) and May ($r=0.906$, $p<0.05$) between the total ABs (sum of all antibiotics (ng/L) for each sampling site) and total ARGs (sum of log(copies/mL) of all ARGs at each sampling site), however there were no significant positive correlations in August. This indicated that when an increased quantity of antibiotics was present in a sample, there generally also seemed to be increased quantities of ARGs, which is not unexpected as ABs can enrich for bacteria which carry ARGs (Martinez, 2009).

In order to assess the effect of individual ABs on ARG levels, Pearson correlation analysis was also performed on ARGs and the ABs they confer resistance to (i.e., *bla*$_{CTX\text{-}M}$, *bla*$_{TEM,}$ and *mecA* confer resistance to amoxicillin, cefaclor, cefprozil, and cefdinir; *qnrS* confers resistance to levofloxacin, and ciprofloxacin; *ermB* confers resistance to azithromycin, clindamycin, and clarithromycin) (Jia et al. 2017). In April there were significant correlations found between *qnrS* and ciprofloxacin ($r=0.726$, $p<0.05$), and *ermB* and clarithromycin ($r=0.936$, $p<0.05$) when all sampling locations were examined ($n=10$); which linked the presence of these ABs and their associated resistance genes. *Int1* also had significant correlations with ciprofloxacin ($r=0.684$, $p<0.05$) and clarithromycin ($r=0.779$, $p<0.05$). Of note, UP2, UP6, and UP7 contained more ABs than the other upstream sites, or even the downstream site. These sites in April often had elevated levels of ARGs, therefore it is possible that increased AB pollution at these sites contributed to increased ARG abundance. Although not significantly correlated, *mecA* levels were together with cefdinir (a beta-lactam antibiotic, *mecA* sensitive) elevated in the upstream samples (especially UP2 and UP7) compared to other samples. This is interesting given that *mecA* was not expected to be found in such high levels in upstream rivers samples, however these spikes in April coincided with high levels of cefdinir. As discussed earlier, the snow melt likely contributed to this sampling event and may have transported ABs into the river that had been preserved during the low temperature season (Dolliver and Gupta, 2008). In May, there were significant ($p<0.05$) correlations between $bla_{CTX\text{-}M}$, $bla_{TEM,}$ and *int1*, and amoxicillin ($r=0.871$; $r=0.675$; $r=0831$), *qnrS* and ciprofloxacin ($r=0.963$), and *ermB*, *int1*, and azithromycin ($r=0.926$; $r=0.816$), which suggested co-occurrences of these ABs and their respective ARGs. In August no significant ($p>0.05$) correlations between ARGs and the ABs they confer resistance to were found. There also seemed to be less ABs (besides amoxicillin) detected in the further upstream samples in August compared to the other months, suggesting a seasonal trend with ABs presence or persistence due to altered environmental conditions (e.g., temperatures of 19-22°C) in the river. These results also suggested that there are potential sources of ABs in the relatively undeveloped upstream watershed.

4 Conclusions

This study assessed the relative impact of the effluent from a tertiary WWTP on the concentration and load of ARGs in a river system draining a largely undeveloped and rural watershed. While meeting strict effluent quality guidelines the WWTP still had a significant impact on ARG levels in the river, although it is not known if and how far downstream the signatures of ARG abundance return to background levels. However, it was found that comparable ARG loads originated from the upstream watershed during wet weather events. These background ARG levels suggest that even areas thought to be undeveloped are likely more affected by anthropogenic activities than originally thought, or that there are larger natural reservoirs of bacteria that harbour ARGs in the environment. The use of additional water quality monitoring tools, such as the human specific *HF183 Bacteroidales* marker, provided important corroborating evidence of ARG sources in this watershed.

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Figure Captions

Figure 1. Map of WWTP and watershed sampling locations detailing land use in the area. Sampling locations are labeled as I and E (influent and effluent), D (downstream), and UP1 to UP7 (Upstream 1 to Upstream 7).

Figure 2. Box plots (minimum to maximum concentrations of all ARGs as well as *int1*) showing seasonal and locational patterns of (a) ARG markers (absolute abundances log (gene copies/mL) in the influent, effluent, and river samples from April, May, and August 2016. 16S rRNA log (gene copies/mL) levels are also shown (filled circles); and (b) ARG loading (log copies/sec) in the influent, effluent, and river samples obtained downstream and upstream from the WWTP. In each plot, the dots represent each ARG marker, while lines represent the median log gene copies/mL of each sampling location. Samples below the LOQ for each ARG were set to ½ LOQ.

Figure 3. Seasonal absolute abundances (log (copies/mL)) for ARGs in the influent (I) and effluent (E) from the WWTP and river water samples obtained downstream (D) and upstream (UP) of the WWTP. Samples below the LOQ (shown as a red dashed line) for each ARG were set to ½ LOQ. Samples below LOD were set to ½ LOD (-0.30 log units) for river samples).

Figure 4. Content of fecal indicator markers in influent and effluent samples from the WWTP and in river water samples downstream and upstream from the discharge point showing: a) *E. coli* values on April, May and August 2016 (LOD 1 MPN/100 mL), and b) *HF183* human *Bacteroidales* marker on April, May and August 2016. *HF183* values were corrected if below

the LOQ (values <LOQ set to ½ LOQ). Samples that appear not to have a bar are <LOD and therefore contained <0.5 *HF183* copies/mL or less.

Tables

Table 1. Description of sampling sites and land use of their corresponding drainage areas.

Sample location	Sample collection dates	Drainage area	Forest including wetland		Agriculture		Wetland		Lakes		# of residences
		km^2	km^2	% of total	km^2	% of total	km^2	% of total	km^2	% of total	
Outlet (include I, E, D, UP1)	Aug.18/ 2015, Feb.23, Apr.12, May 10, Aug.2/ 2016	116	106	91.8	2.1	1.8	3.7	3.2	3.8	3.3	223
UP2	Apr.12, May 10, Aug.2 /2016	114	106	92.6	1.8	1.6	3.7	3.2	3.8	3.4	81
UP3	Apr.12, May 10, Aug.2/ 2016	111	104	94.1	0.5	0.5	3.7	3.3	3.8	3.5	41
UP4	Apr.12, May 10, Aug.2/ 2016	109	103	94.6	0.4	0.4	3.7	3.4	3.8	3.5	21
UP5	Apr.12, May 10, Aug.2/ 2016	46	44	95.4	0.0	0.0	1.9	4.0	2.0	4.3	2
UP6	Apr.12, May 10, Aug.2/ 2016	3.9	3.7	93.7	0.1	1.8	0.1	1.9	0.0	0.0	1
UP7	Apr.12, May 10, Aug.2/ 2016	12	11	98.9	0.0	0.0	1.3	11	0.1	0.9	0

Figures

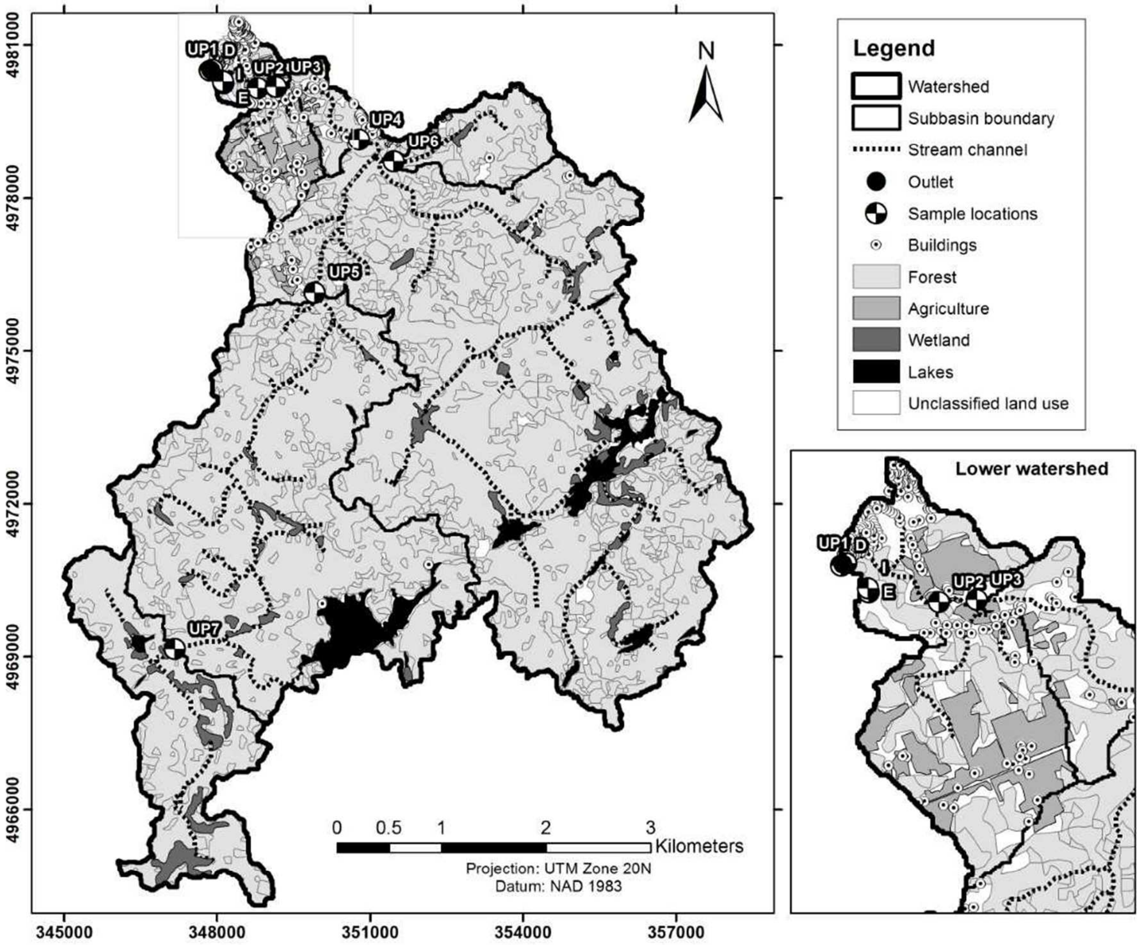
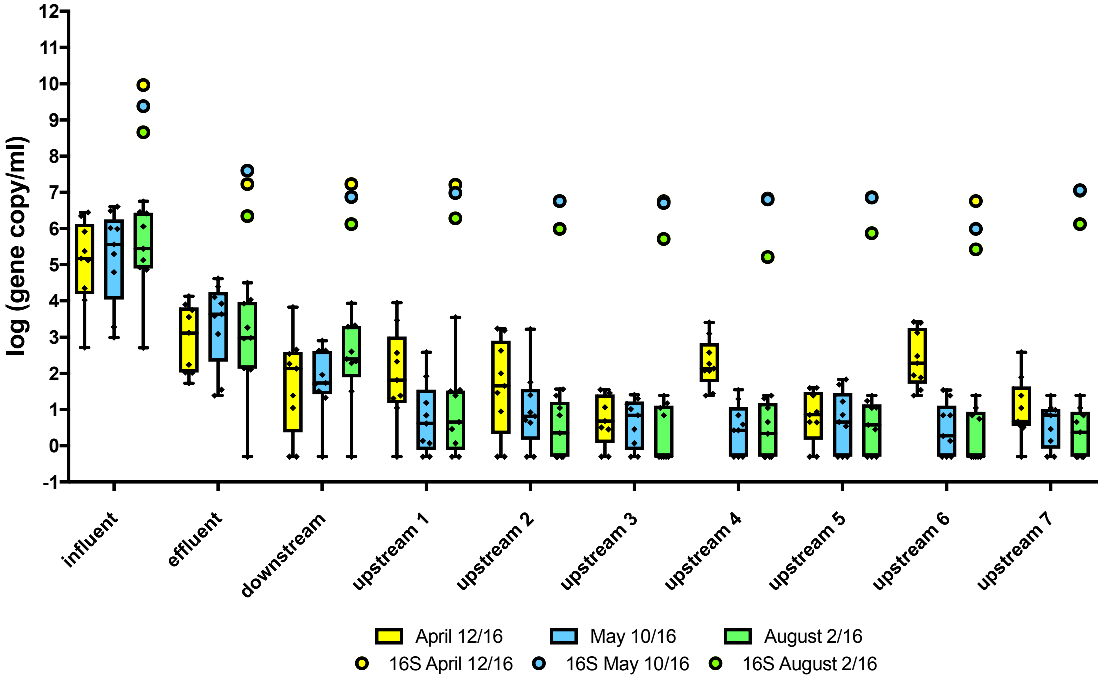


Figure 1

a)


log (gene copy/ml)
12
11
10
9
8
7
6
5
4
3
2
1
0
-1
influent
effluent
downstream
upstream 1
upstream 2
upstream 3
upstream 4
upstream 5
upstream 6
upstream 7
April 12/16
May 10/16
August 2/16
16S April 12/16
16S May 10/16
16S August 2/16

b)

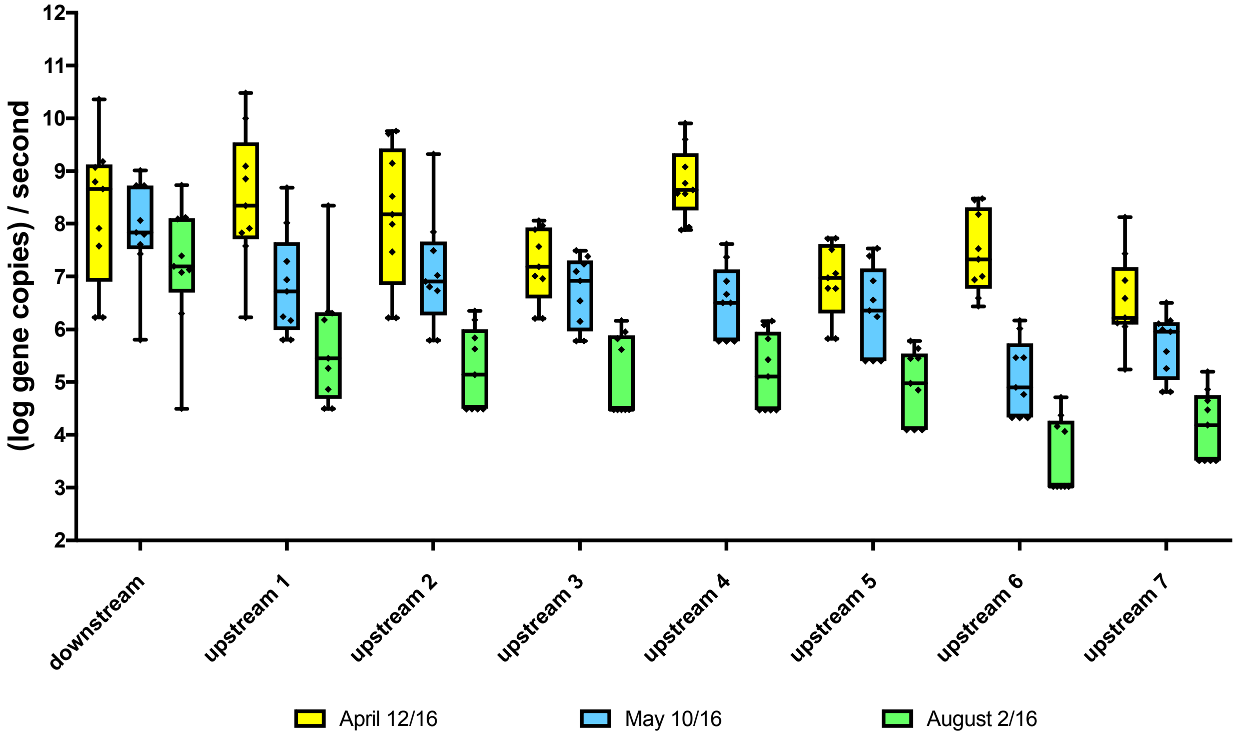

(log gene copies) / second
12
11
10
9
8
7
6
5
4
3
2
downstream
upstream 1
upstream 2
upstream 3
upstream 4
upstream 5
upstream 6
upstream 7
April 12/16
May 10/16
August 2/16

Figure 2

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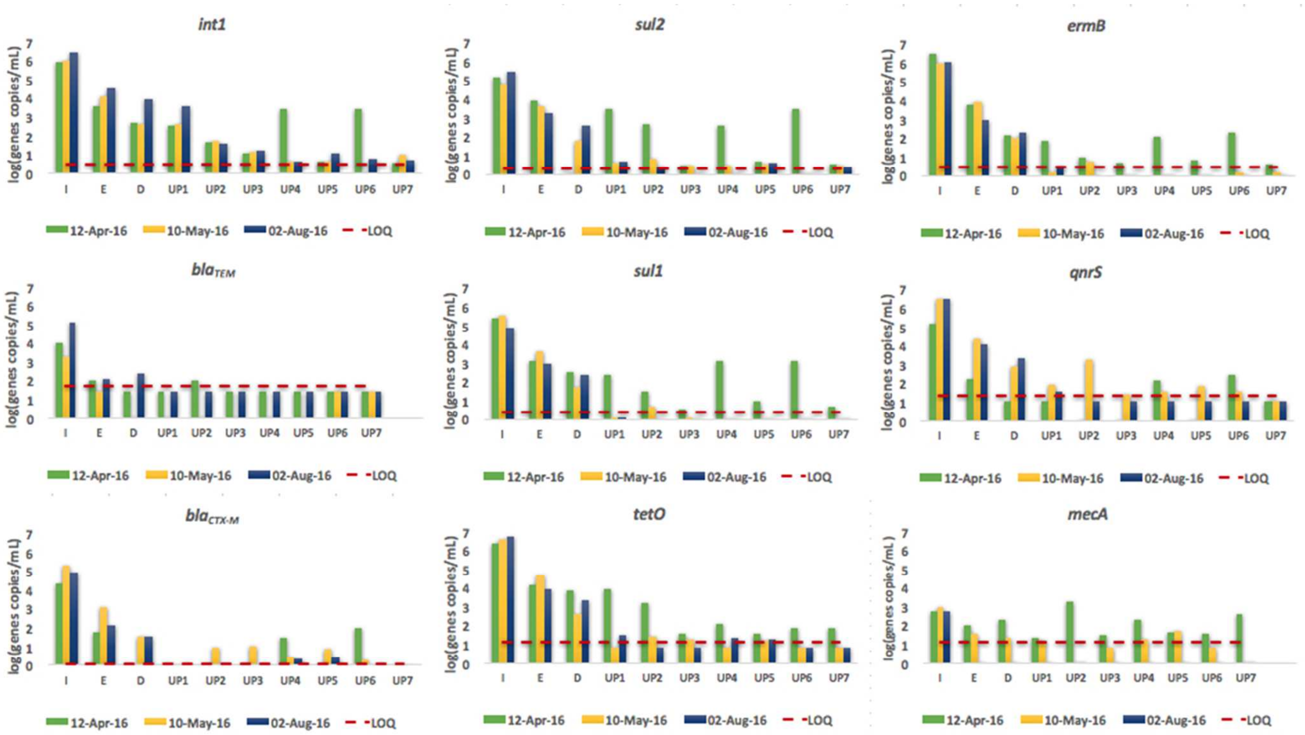


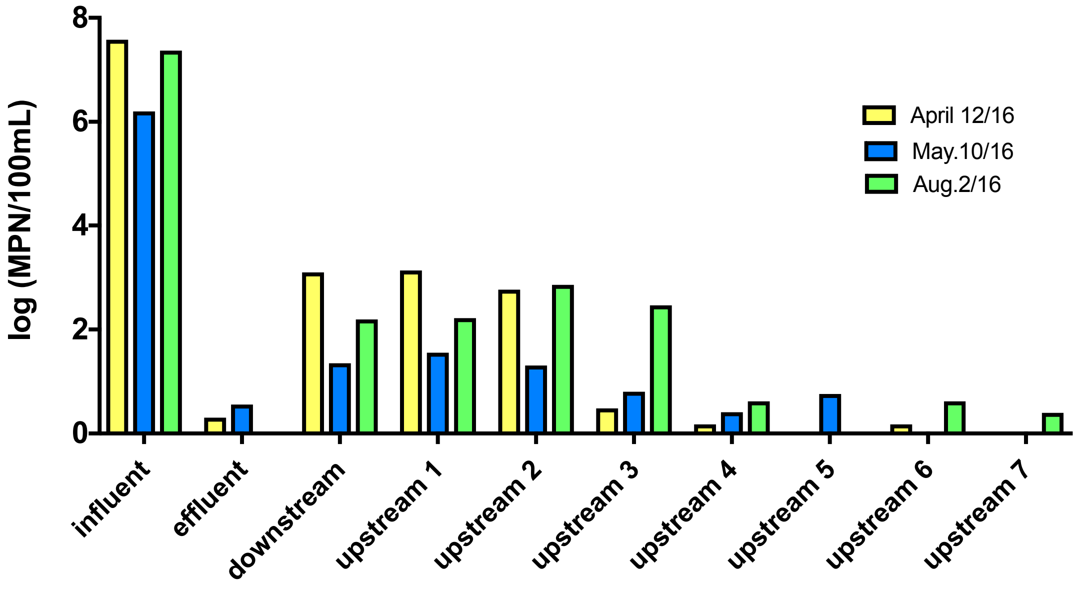
695

696 Figure 3

697

698

a)


log (MPN/100mL)
8
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influent
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downstream
upstream 1
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upstream 6
upstream 7
April 12/16
May.10/16
Aug.2/16

b)

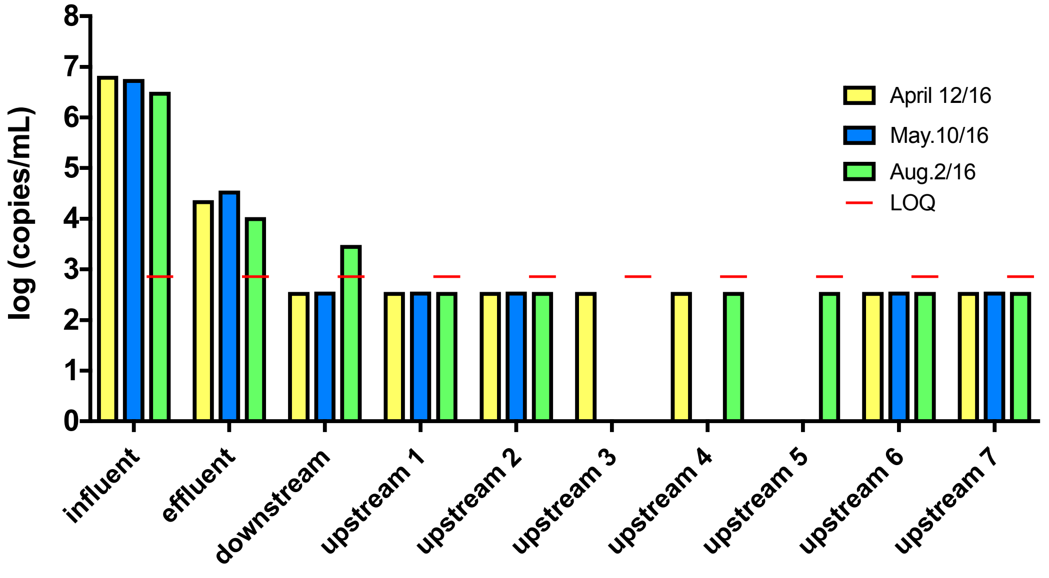
log (copies/mL)
8
7
6
5
4
3
2
1
0
influent
effluent
downstream
upstream 1
upstream 2
upstream 3
upstream 4
upstream 5
upstream 6
upstream 7
April 12/16
May.10/16
Aug.2/16
LOQ

Figure 4.

Supporting Material

Table S1. Quantitative PCR primer sequences and reaction conditions

Target gene	Primer	Sequence (5'-3')	Conditions	Reference
16S rRNA	1369F 1492R	CGGTGAATACGTTCYCGG GGWTACCTTGTTACGACTT	95°C for 10 mins; 40 cycles of 95°C for 15s, 55°C for 30s, and 72°C for 30s	Suzuki et al., 2000
erm(B)	ermBF ermBR ermBP	GGATTCTACAAGCGTACCTTGGA GCTGGCAGCTTAAGCAATTGCT FAM-CACTAGGGTTGCTCTTGCACACTCAAGTCBHQ-1	95°C for 3 min; 40 cycles of 95°C for 15s, 62°C for 30s	Böckelmann et al., 2009
MecA	mecAF mecAR mecAP	CATTGATCGCAACGTTCAATTTAAT TGGTCTTTCTGCATTCCTGGA FAM-CTATGATCCCAATCTAACTTCCACATACCBHQ-1	95°C for 3 min; 40 cycles of 95°C for 15s, 62°C for 30s	Böckelmann et al., 2009; Francois et al., 2003
tetO	tetOF tetOR tetOP	AAGAAAACAGGAGATTCCAAAACG CGAGTCCCCAGATTGTTTTTAGC FAM-ACGTTATTTCCCGTTTATCACGGAAGCG-BHQ-1	95°C for 3 min; 40 cycles of 95°C for 15s, 62°C for 30s	Böckelmann et al., 2009
bla$_{CTX\text{-}M}$	*BLACTX-M* UP *BLACTX-M* LP CTX-probe	ACCAACGATATCGCGGTGAT ACATCGCGACGGCTTTCT FAM-TCGTGCGCCGCTG-BHQ1	95°C for 3 min; 40 cycles of 95°C for 15s, 62°C for 30s	Colomer-Lluch et al., 2011
bla$_{TEM}$	TEM UP TEM LP TEM Probe	CACTATTCTCAGAATGACTTGGT TGCATAATTCTCTTACTGTCATG FAM-CCAGTCACAGAAAAGCATCTTACGG-BHQ1	95°C for 3 min; 40 cycles of 95°C for 15s, 62°C for 30s	Lachmayr et al., 2008
sul1	qSUL653f qSUL719r tpSUL1	CCGTTGGCCTTCCTGTAAAG TTGCCGATCGCGTGAAGT FAM -CAGCGAGCCTTGCGGCGG-BHQ1	95°C for 3 min; 40 cycles of 95°C for 15s, 62°C for 30s	Czekalshi et al., 2012; Heuer et al., 2008
sul2	qSUL2_595f qSUL2_654r tpSUL2_614	CGGCTGCGCTTCGATT CGCGCGCAGAAAGGATT FAM -CGGTGCTTCTGTCTGTTTCGCGC-BHQ1	95°C for 3 min; 40 cycles of 95°C for 15s, 62°C for 30s	Czekalshi et al., 2012; Heuer et al., 2008
qnrS	*qnrS* UP *qnrS* LP *qnrS* probe	CGACGTGCTAACTTGCGTGA GGCATTGTTGGAAACTTGCA FAM –AGTTCATTGAACAGGGTGA-BHQ1	95°C for 3 min; 40 cycles of 95°C for 15s, 62°C for 30s	Colomer-Lluch et al., 2014
VanA	vanAF vanAR vanAP	CTGTGAGGTCGGTTGTGCG TTTGGTCCACCTCGCCA FAM-CAACTAACGCGGCACTGTTTCCCAAT-BHQ-1	95°C for 3 min; 40 cycles of 95°C for 15s, 62°C for 30s	Volkmann et al., 2004
int1	intI1-LC1 intI1-LC5 intI1-probe	GCCTTGATGTTACCCGAGAG GATCGGTCGAATGCGTGT FAM-ATTCCTGGCCGTGGTTCTGGGTTTT-BHQ1	95°C for 3 min; 40 cycles of 95°C for 15s, 62°C for 30s	Barraud et al., 2010
HF183	HF183-F HF183-R HF183-p	ATCATGAGTTCACATGTCCG CTTCCTCTCAGAACCCCTATCC CTAATGGAACGCATCCC	95°C for 3 min; 40 cycles of 95°C for 30s, 58°C for 30s	Haugland *et al*., 2010; Layton *et al*., 2013

Table S2. Average and maximum effluent water quality from sampling events (n=5, August 18 2015 and February 23, April 12, May 10, August 2 2016)

Parameter	**Average**	**Max**
$CBOD_5$ (mg/L)	2.051	3.64
TSS (mg/L)	1.225	1.6
VSS (mg/L)	0.913	1.25
COD (mg/L)	13.68	20.00
TN (mg/L)	16.08	27.2
TP (mg/L)	2.84	8.5
Nitrate (mg/L)	11.58	16.9
Ammonia (mg/L)	0.414	0.887
Total coliforms (MPN/100mL)	1.304	2.273
E. coli log(MPN/100mL)	0.363	0.556

Table S3. Antibiotics detected in samples (ng/L) in water samples obtained on April 12, May 10, and August 2, 2016.

Sample	**Month (2016)**	**Amox-icillin**	**Cefa-clor**	**Cef-dinir**	**Levo-floxacin**	**Cipro-floxacin**	**Azithro-mycin**	**Clinda-mycin**	**Clarithro-mycin**	**Triclo-carban**
April MDL[‡] (ng/L)		**16**	**542**	**50**	**28**	**63**	**39**	**1**	**1**	**28**
May/August MDL (ng/L)		**35**	**29**	**95**	**91**	**71**	**40**	**30**	**74**	**50**
Influent	April	<DL	<DL	<DL	156	2246	196	13	406	48
	May	3949	<DL	<DL	<DL	383	300	33	115	158
	August	<DL	77	<DL	<DL	<DL	<DL	<DL	<DL	75
Effluent	April	<DL	<DL	<DL	78	747	54	36	181	<DL
	May	4600	<DL	<DL	<DL	201	342	37	192	65
	August	<DL	<DL	<DL	99	195	<DL	<DL	<DL	68
Down-stream	April	<DL	<DL	<DL	<DL	593	<DL	5	6	<DL
	May	741	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL
	August	739	<DL	<DL	<DL	<DL	82	<DL	<DL	<DL
Upstream 1	April	<DL	<DL	<DL	29	581	<DL	3	1	<DL
	May	674	<DL	<DL	<DL	91	84	<DL	<DL	<DL
	August	634	<DL	<DL	<DL	259	42	<DL	<DL	56
Upstream 2	April	<DL	<DL	535	173	969	<DL	4	7	<DL
	May	743	<DL	<DL	<DL	<DL	113	<DL	<DL	<DL
	August	648	<DL	<DL	<DL	72	40	<DL	<DL	<DL
Upstream 3	April	<DL	<DL	<DL	<DL	576	<DL	3	2	<DL
	May	903	<DL	<DL	<DL	<DL	105	<DL	<DL	<DL
	August	597	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL
Upstream 4	April	<DL	<DL	<DL	<DL	600	<DL	4	1	<DL
	May	798	<DL	<DL	<DL	<DL	118	<DL	<DL	<DL
	August	622	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL
Upstream 5	April	<DL	<DL	<DL	<DL	560	<DL	4	1	<DL
	May	772	<DL	<DL	<DL	<DL	97	<DL	<DL	<DL
	August	1032	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL
Upstream 6	April	<DL	<DL	844	33	606	<DL	5	1	<DL
	May	410	<DL	<DL	<DL	120	120	<DL	<DL	55
	August	639	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL
Upstream 7	April	<DL	<DL	211	67	734	<DL	4	1	<DL
	May	696	<DL	<DL	<DL	<DL	90	<DL	<DL	58
	August	766	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL

[‡] Detection Limit (DL). Cefprozil consistently not detected above the detection limit (DL) and was omitted from the table.