Seasonal dynamics of tetracycline resistance gene transport in the Sumas River agricultural watershed of British Columbia, Canada

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Keen et al. (2018) ***Science of the Total Environment*** **628-629: 490-8**

ABSTRACT:

Environmental transport of contaminants that can influence the development of antibiotic resistance in bacteria is an important concern in the management of ecological and human health risks. Agricultural regions are locales where practices linked to food crop and livestock production can introduce contaminants that could alter the selective pressures for the development of antibiotic resistance in microbiota. This is important in regions where the use of animal manure or municipal biosolids as waste and/or fertilizer could influence selection for antibiotic resistance in pathogenic bacterial species. To investigate the environmental transport of contaminants that could lead to the development of antibiotic resistance in bacteria, a watershed with one of the highest levels of intensity of agricultural activity in Canada was studied; the Sumas River located 60 km east of Vancouver, British Columbia. This two-year assessment monitored four selected tetracycline resistance genes (*tet*(O), *tet*(M), *tet*(Q), *tet*(W)) and water quality parameters (temperature, specific conductivity, turbidity, suspended solids, nitrate, phosphate and chloride) at eight locations across the watershed. The tetracycline resistance genes (Tc^r) abundances in the Sumas River network ranged between 1.47×10^2 and 3.49×10^4 copies/mL and ranged between 2.3 and 6.9 copies/mL in a control stream (located far from agricultural activities) for the duration of the study. Further, Tc^r abundances that were detected in the wet season months ranged between 1.3×10^3 and 2.29×10^4 copies/mL compared with dry season months (ranging between 0.6 and 31.2 copies/mL). Highest transport rates between 1.67×10^{11} and 1.16×10^{12} copies/sec were observed in November 2005 during periods of high rainfall. The study showed that elevated concentrations of antibiotic resistance genes in the order of $10^2 – 10^4$ copies/mL can move through stream networks in an agricultural watershed but seasonal variations strongly influenced specific transport patterns of these genes.

KEYWORDS: antibiotic resistance genes; tetracycline; environment; transport; agriculture; seasonality.

1 INTRODUCTION

There is ever-growing concern over the role of the transport and distribution of environmental contaminants that create selective pressures at the genetic level to develop antibiotic resistance in indigenous bacteria. As such, receiving environments are now recognized as both a source and reservoir of genetic determinants of resistance (Alonso et al., 2001; Wright, 2007; Aminov, 2009; Levy, 2012) that can spread throughout wider environmental compartments extending the opportunities for genetic exchange of antibiotic resistance genes (ARGs) between bacteria (Séveno et al., 2002; Martinez, 2008; Davies and Davies, 2010). Some antibiotics and their resistance genes originate in nature (Cantón, 2009; Martinez, 2009); however, low levels of some environmental pollutants can alter selection pressures (Baquero and Coque, 2014) to enrich populations of antibiotic resistant bacteria and increase the risk to public health (Zdziarski et al., 2003; Pruden, 2014; Huijbers et al., 2015). Moreover, environmental contamination with trace metals can play a role in co-selection of antibiotic resistance in bacteria found in soil and water ecosystems (Baker-Austin, 2006; Seiler and Berendonk, 2012). Environmental contaminant transport affects processes that regulate the spread of ARGs (Kim et al., 2010) and there is an on-going need to better understand the fate and dynamics of antibiotic resistance in ecosystems (Suzuki et al., 2017). Evidence demonstrates that contaminant exposure can cause genetic stress responses that allow ARGs to be readily exchanged between pathogens and indigenous bacteria present in surface water, groundwater, biofilms, sediments and soils (Mackie et al., 2006;

65 Baquero et al., 2008; Allen et al., 2010). Seasonal variability of non-point source pollutants in
66 agricultural watersheds has been described (Udawatta et al., 2006; Poudel et al., 2013; Zhou et
67 al., 2014).

68 Therefore, it is important to understand the seasonal variability in the flux of contaminants that
69 could serve as indicators for potential presence of antibiotic resistant bacteria in agricultural
70 regions particularly when the intensity and frequency of rainfall events changes seasonally. Use
71 of antibiotics in veterinary medicine or in fruit tree pest control is an important source of
72 agricultural drug residues (Kümmerer, 2009; McManus et al., 2002; Christiano et al. 2010).
73 Metabolic processes often do not affect the biological activity of antibiotics, and thus land
74 application of animal waste increases the likelihood of contaminant transport (Sarmah et al.,
75 2006). This situation can be amplified by low levels of antibiotics used for the purposes of
76 prophylaxis or growth promotion of food animals (Ghosh and LaPara, 2007). The presence of
77 antibiotic compounds and ARGs in the environment related to agricultural practices have been
78 documented (Halling-Sørensen et al., 1998; Yang and Carlson, 2003; McKinney et al., 2010);
79 however, larger scale combined temporal-spatial studies of resistance gene transport remain
80 limited.

81 This paper describes the results of a two-year study conducted 2004–2006 that examined the
82 seasonal dynamics and environmental transport of tetracycline resistance (Tc^r) genes (as
83 exemplars) in an agricultural watershed and stream network located near Vancouver, British
84 Columbia, Canada. The overarching hypothesis was that abundances of Tc^r genes are higher in
85 stream networks within agricultural regions and the transport of such genes is influenced by
86 seasonal patterns. Quantitative PCR (qPCR) was used to measure relative abundance of four

87 common Tc^r determinants (*tet*(O), *tet*(M), *tet*(Q), *tet*(W)) and 16S rRNA genes in bacteria from
88 water samples collected 20 cm beneath the water surface on a monthly basis. In addition,
89 standard water quality parameters including temperature, specific conductivity, turbidity,
90 suspended solids, nitrate, phosphate and chloride were monitored in the receiving water bodies to
91 investigate possible correlations of key indicators of contamination with the observed abundance
92 of the selected tetracycline resistance genes. The potential for transport of Tc^r genes along a
93 defined segment of the Sumas River was examined by measuring the flow rate and calculating
94 mass flux of the specific indicator genes under varying stream discharge conditions.

95 **2 MATERIALS AND METHODS**

96 ***2.1 Study Location***

97 This study was conducted in the Sumas watershed located 60 km east of Vancouver, British
98 Columbia, Canada between July 2004 and March 2006. The watershed represents approximately
99 5,700 hectares of one of the most economically important areas in Canada for production of
100 poultry, dairy, hogs, fruit and vegetable and nursery farms (Schreier et al., 2001). The segment
101 of the Sumas River investigated here is a second order stream (branch of the Fraser River system
102 which eventually discharges into the Pacific Ocean) that flows northward towards the Fraser
103 River from the Canada – US border (Figure 1). Some tributaries and canals feed into the Sumas
104 River; eight sampling sites were chosen along an approximately 23 km stretch of the Sumas
105 River stream network at elevations that ranged between 7 and 11 m above sea level
106 (Environment Canada Topographic Data, 2017). The study location was specifically selected to
107 represent agricultural activities in the region with all 8 sampling stations located within 200 m of
108 dairy farms, poultry barns or field food crops. In addition to these sample sites, one control site,

situated at 1,050 m above sea level on a stream flowing from a forested headwater on the eastern boundary of the watershed (approximately 8 km east of the study location), was designated as a reference site where chemical analyses of chloride, nitrate, phosphate and trace metals revealed negligible possibility of urban or agricultural influence on water quality.

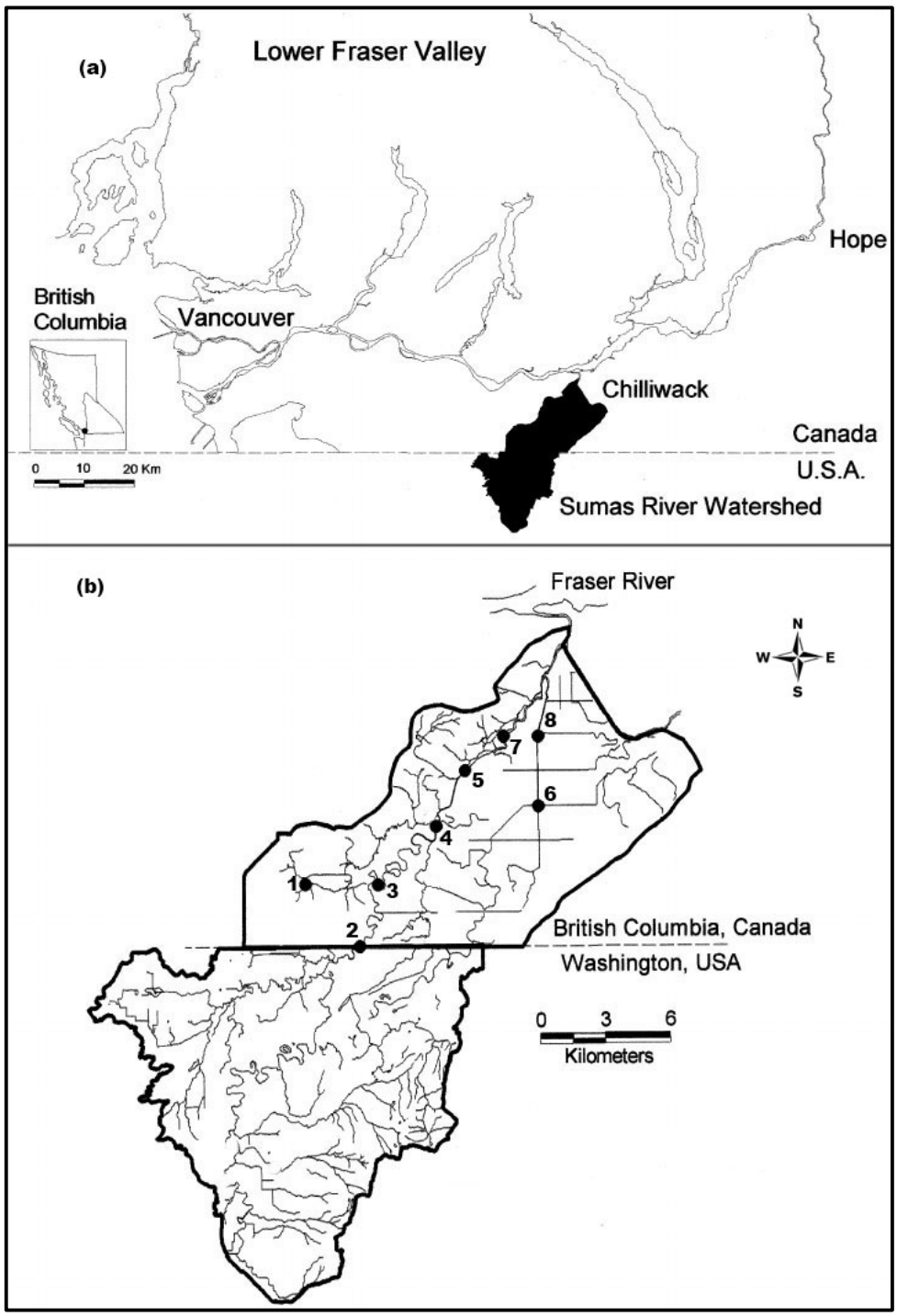


Figure 1: Map showing (a) geographic location of Sumas River Watershed in British Columbia, Canada with (b) sampling site locations (sites 2–5 used for flux measurements). Map used with permission (Berka et al., 2001)

2.2 Sample collection and processing

Samples for molecular microbial analyses (in quadruplicate) were collected in acid-washed, autoclaved 250 mL amber glass bottles and stored on ice prior to returning to the laboratory. In total, 684 samples were collected and analyzed on an approximately monthly basis over the course of the study period. Field measurements of temperature and conductivity (reported as specific conductivity) were determined at each sampling station using a Yellow Springs Instrument (YSI) Model #30M/50 meter. Dissolved oxygen was measured *in situ* with an YSI Model #58 portable meter and turbidity was measured using a Hach model 2100P portable turbidometer.

2.3 DNA Extraction

Samples for molecular microbial analyses (transported on ice) were filtered within two hours of sample collection with the filters immediately frozen on dry ice. The filters were shipped within 24hrs to the Department of Civil, Architectural and Environmental Engineering at the University of Kansas, Lawrence, Kansas where they remained frozen at -20^{o}C until extraction which was conducted monthly between July 2004 and March 2006. One hundred mL volumes of samples were filtered through pre-sterilized 0.22 μm porosity Nalgene disposable filter funnels (NNI, Rochester NY). Filters from replicates were extracted using MoBio UltraClean Soil DNA kits (Solona Beach, CA) with minor method modifications recommended by the kit manufacturer (MoBio Laboratories, 2004). Filters, beads and extraction buffers were combined, homogenized for 30 seconds (speed 5.5) using a FastPrep (Qbiogene, Irvine, CA) cell disruptor and then incubated at 70^{o}C for 10 minutes to enhance lysis of Gram-positive bacteria. Following incubation, samples were re-agitated for 30 seconds (speed 4.5) and subjected to the further purification steps of the kit manufacturer's protocol. All resulting 50 μL extracts were stored at -

141 20^{o}C prior to analysis. Three replicates were analysed by qPCR and one sample replicate was
142 saved at -80^{o}C for archival purposes.

143 ***2.4 qPCR assays***

144 Four common Tc^{r} genes ((*tet*(M), *tet*(O), *tet*(Q), and *tet*(W)) and 16S rRNA genes were selected
145 for quantification by qPCR analyses. These four Tc^{r} genes were specifically chosen for
146 comparison with parallel studies examining antibiotic resistance in a US feedlot (Peak et al.,
147 2007; Engemann et al., 2008). The TaqMan probe/primer sets and the plasmid standards used in
148 this investigation have been described previously for *tet*(M) (Peak et al., 2007), *tet*(O), *tet*(Q) and
149 *tet*(W) (Smith et al., 2004) and for 16S-rRNA gene (Harms et al., 2003). Sample aliquots of 2
150 µL DNA templates were mixed with iQ Supermix PCR reagents (BioRad, Hercules, CA) and
151 500 nM of each primer. A BioRad iCycler equipped with an iCycler iQ fluorescence detector
152 was used for the reactions. Standard curves were constructed from quantification of copy
153 numbers for each gene prepared by 10 –fold serial dilution of the extracted samples for
154 appropriate plasmid DNA that ranged from 1.0 to 1 x 10^{7} copies per reaction in order to optimize
155 PCR reactions and minimize impact of inhibitors that may have been carried over during DNA
156 extraction (Smith et al., 2004). The qPCR efficiencies ranged between 95–105 % as determined
157 by comparing serial dilutions of some selected samples using the 16S-rRNA assay. Reaction
158 specificities were verified using melt curves (55–95^{o}C). All samples were analysed in triplicate
159 and reported abundances represent the arithmetic mean of three measurements.

160

161 ***2.5 Water Quality Analyses***

Total solids (for determining dry weight sample equivalents where necessary) were measured using method 2540 B (dried at 105°C) (APHA, 1995) and suspended solids were measured by method 2540 C (dried at 105°C) (APHA, 1995). Chloride concentration was measured by mercuric thiocyanate flow injection analysis using standard method 4500-Cl^- G (APHA, 1995). Samples collected for nitrate/nitrite and phosphate measurements were preserved in the field immediately with 0.1 g/100 ml phenylmercuric acetate in 20% (v/v) acetone. Cadmium reduction flow injection analysis was used to measure nitrate/nitrite using standard method 4500-NO_3^- I (APHA, 1995) and phosphate was measured by flow injection analysis for orthophosphate using method 4500-P G (APHA, 1995). Only for the pre-screening for suitability of candidate control sites, 29 – element ICP – OES (inductively coupled plasma optical emission spectrometry) scans were conducted using an Optima 7300 V ICP-OES (Perkin Elmer).

2.6 Estimating Transport of Antibiotic Resistance Genes

In order to quantify the mass flux of the four Tc^r genes along a specified ~12.6 km segment of the watercourse network within the Sumas watershed (see Figure 1b), stream velocities were measured monthly at four sentinel stations (sampling sites 2, 3, 4 and 5) between July and December 2005. A Swoffer Model 3000 flow meter was used to measure the velocities at 3-5 points across the cross-section of the stream (either by wading across the width of the stream or using an inflatable canoe when stream depth increased) at varying depths (Site 2: 0.47–1.13 m; Site 3: 0.77–0.95 m; Site 4: 0.72–1.07 m; Site 5: 0.39–0.51 m). Volumetric flow (Q) was calculated using the equation Q (m^3/sec) = vA and assuming uniform velocity (v) at 0.3 times depth (Chanson, 2003) and that depth was uniform across estimated cross-sectional area (A).

2.7 Data Analyses

All Tc^r quantification values were normalized to 16S rRNA gene abundances to account for the differences in background bacterial abundances and for any variations in the extraction efficiencies. Statistical analyses of the differences between Tc^r gene abundances were performed using SPSS (v 13.01, Chicago, IL) data analysis software. The data were log-transformed with means and 95% confidence intervals calculated for use as the statistical descriptors for resistance gene abundances. The differences between the means were assessed by paired Mann-Whitney U tests and were considered significant at $p < 0.05$. Observations described as occurring during dry season were measured between April and October and those described as occurring in wet season were measured between November and March.

Spearman's Rank correlations (2-tailed) were calculated using SPSS statistical software Version 13.01 to compare water quality parameters with tetracycline resistance gene abundances over the period of July 2004 and March 2006.

3 RESULTS

3.1 Quantification of Tetracycline Resistance Genes

All four of the selected Tc^r genes were detected at the sampling locations along the segment of the Sumas River during every month that was monitored (Figure 2) although the proportion of each gene relative to the total gene abundance was variable (Figure 3). On five sampling occasions (Nov 8, 2004; Dec 2, 2004; Apr 28 2005; Nov 1, 2005 and Feb 10, 2006), Tc^r genes were also detected at the reference control site although the measured abundance of total Tc^r genes was very low (2.3–6.9 gene copies/mL). Microbial abundance, as indicated by

measurement of concentration of 16S rRNA genes, ranged between 5.81 x 10^5 and 1.93 x 10^7 copies/mL among all stream sites during the monitoring period and was significantly higher (p < 0.05) than that at the control site (ranging between 4.47 x 10^4 and 5.21 x 10^4 copies/mL).

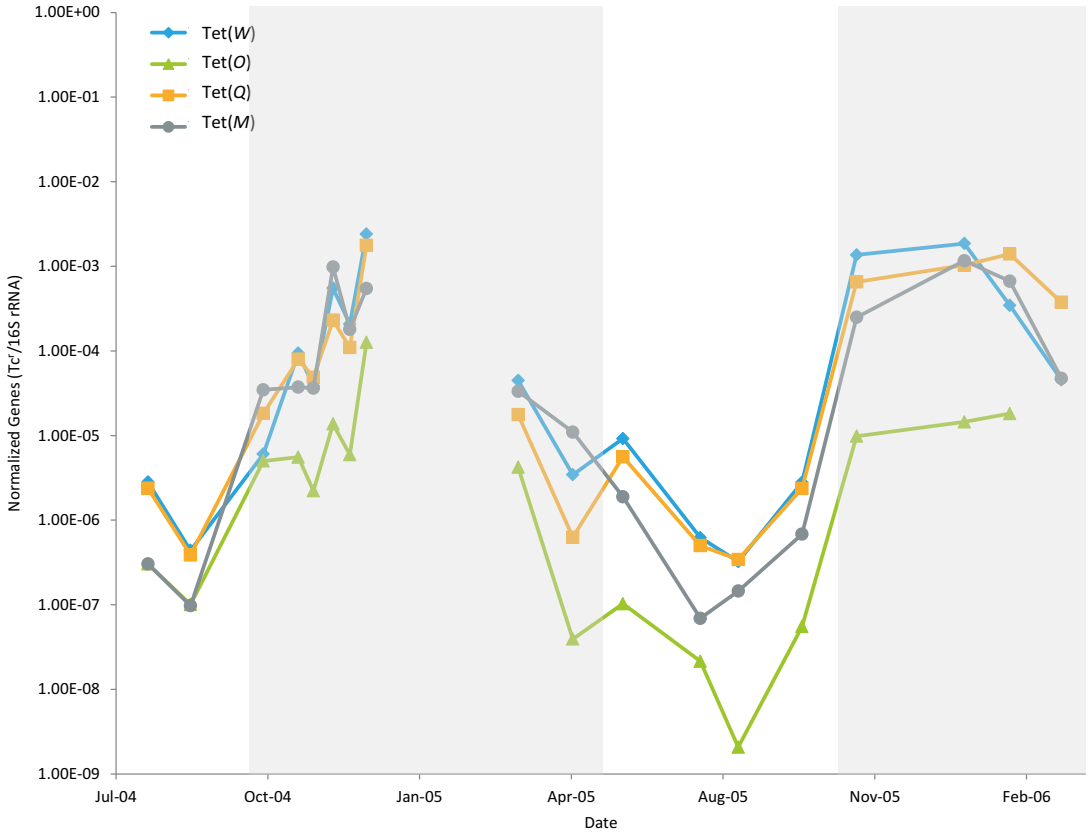
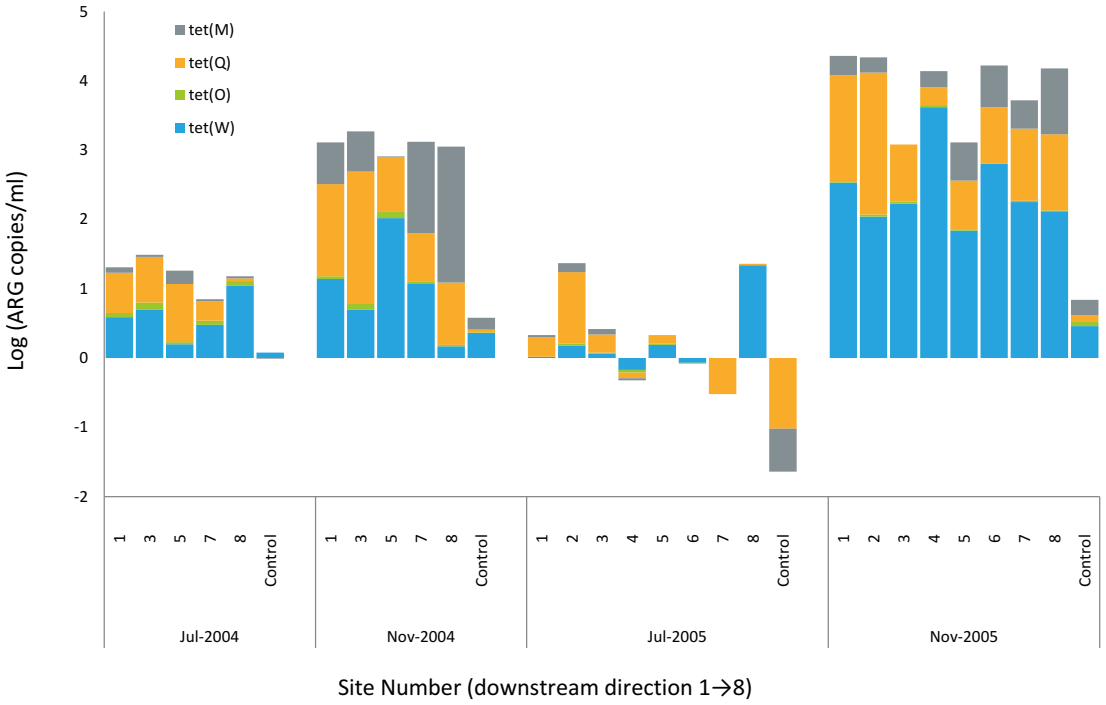


Figure 2: Average Tc^r gene relative abundance (Tc^r gene copies per 16S rRNA gene copies) for the sampling sites for the period of July 2004 – March 2006. Relative abundances of Tc^r genes were significantly higher (p<0.05) in November 2004 and November 2005 than in other months. Sites were not monitored in Jan and Feb 2005. Shaded areas represent wet season.

In the Sumas stream network, seasonal trends of Tc^r gene relative abundances were observed (Figure 2) and occurred similarly each year. The four measured Tc^r genes were normalized to 16S rRNA and the average was calculated for all sites along the river segment to demonstrate the observed trend. The lowest gene abundances were observed during the dry season ranging between 3.5 and 28.6 copies/mL in 2004 and ranging between 0.6 and 31.2 copies/mL in 2005. Higher abundance ($p < 0.05$) of total Tc^r genes occurred in wet season months of November ($1.3 \times 10^3 - 2.15 \times 10^4$ copies/mL in 2004; $4.76 \times 10^3 - 2.29 \times 10^4$ copies/mL in 2005) and December ($4.89 \times 10^2 - 2.26 \times 10^3$ copies/mL in 2004; $1.47 \times 10^2 - 4.51 \times 10^2$ copies/mL in 2005) and in January to March 2006 ($1.16 \times 10^3 - 3.49 \times 10^4$ copies/mL), representing sampling events that coincided with periods of unusually high precipitation in the Sumas watershed region. For comparison purposes, the official stream discharge rates and rainfall measurements recorded by Environment Canada for the study period are presented in Figure 4.

Figure 3 illustrates the abundance profile for the individual Tc^r genes for two specific months of both 2004 and 2005. The proportion of the individual genes relative to the total gene abundance varied from month to month during the study period although some trends were observed. In general, the gene profile was dominated by Tet(*W*), Tet(*Q*) and Tet(*M*) with the abundance of Tet(*O*) being much smaller throughout the duration of the study. November was selected as an example wet season month to demonstrate that despite differences in rainfall and stream discharge rate (Figure 4), some similarity of the Tc^r gene profile could be observed for each consecutive year. July was selected as an example of a dry season month in which no general trend of the abundance profile for individual Tc^r genes could be discerned although the total number of genes measure was low.



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241 Figure 3: Tetracycline resistance gene abundance profile (average of three replicates) for sites in
242 the Sumas River stream network comparing July and November in 2004 and 2005. Relative
243 composition (% total) is represented by segments within each bar (with bar height representing
244 100% of the log-transformed sum of the four Tc^r genes).

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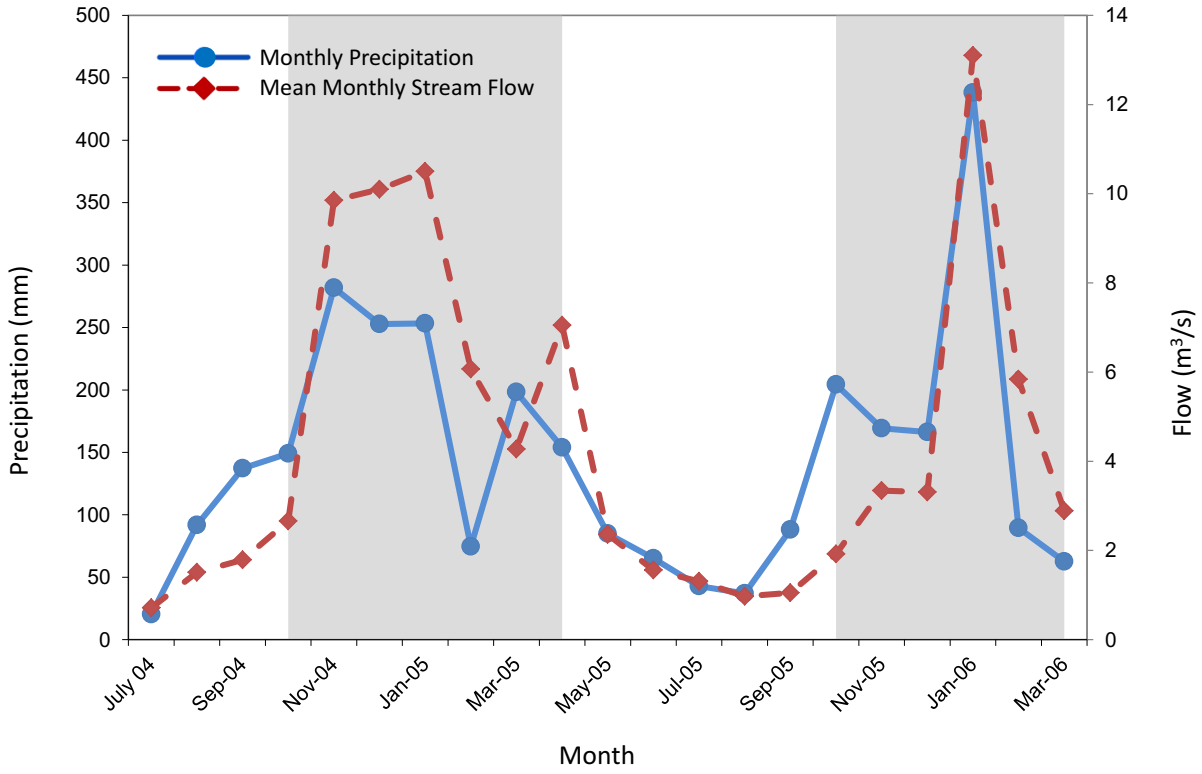

Figure 4: Total monthly precipitation and mean monthly stream discharge in Sumas watershed (Environment Canada Climate Weather Data; accessed May 2016). Shaded areas represent wet season.

3.2 Water Quality Measurements

Measurements of water quality are summarized in Table 1. Specific conductivity, nitrate, and chloride were significantly higher in the Sumas River network ($p < 0.05$) than in the control stream for both wet season months and dry season months while orthophosphate was significantly higher during the wet season months. The standard method used for analyses of nitrogen species in water samples measured both the nitrite and nitrate combined. Nitrite is

rarely measured in appreciable concentrations in surface water samples (Ellis, 1989) and thus all reported concentrations represent nitrate concentrations.

Table 1: Concentration of different physico-chemical variables and total tetracycline resistance genes measured during wet and dry seasons (* indicates statistical significant differences between the Sumas River network and the control stream at $p < 0.05$).

		Wet Season	Control	Dry Season	Control
Temperature (^{o}C)	*average*	7.7	6.4	19.8	18.7
	Std dev	2.6	2.5	4.6	4.3
Dissolved Oxygen (mg/L)	*average*	7.8	12.5	9.1	10.5
	Std dev	2.3	1.1	3.3	1.8
Specific Conductivity (µS/cm)	*average*	236*	72	308*	138
	Std dev	64.5	67.3	24.7	17.1
Turbidity (NTU)	*average*	19.5	8.1	5.0	1.4
	Std dev	19.3	5.9	4.5	1.2
Suspended Solids (mg/L)	*average*	15.4	14.1	4.9	2.3
	Std dev	13.0	10.0	4.5	2.1
NO_x-N (mg/L)	*average*	3.3*	0.4	2.6*	0.7
	Std dev	1.2	0.06	1.3	0.6
PO_4-P (µg/L)	*average*	99.1*	4.89	31.7	6.5
	Std dev	13.0	0.3	25.3	5.9
Cl- (mg/L)	*average*	11.6*	1.0	14.7*	0.7
	Std dev	3.9	0.5	2.1	0.2
Total Tc^r Genes (copies/mL)	*average*	5540*	0.48	11.4	2.95
	Std dev	6564	0.45	17.8	2.60

Certain statistical relationships (Spearman's rank correlations) exist between Tc^r gene abundances and relative Tc^r gene abundances (normalized to 16S rRNA) and selected water quality parameters, stream discharge and precipitation for sites located along the main stream

channel of the Sumas River. Spearman rank correlations were positive ($p < 0.05$) between both Tc^r genes (absolute and relative abundances) and instantaneous discharge, 48 h discharge, 72 h rainfall and turbidity (Table 2) during the monitoring period (illustrated in Figure 4). The range of Spearman's rho calculated for the positive correlations for each of the sites along the stream network comparing abundance of Tc^r genes with instantaneous discharge was $0.762<rho<0.810$; comparing abundance of Tc^r genes with 48 hour discharge was $0.653<rho<0.810$; comparing abundance of Tc^r genes with 72 hour rainfall was $0.502<rho<0.561$; and comparing abundance of Tc^r genes with turbidity was $0.577<rho<0.800$. Significant negative correlations were established for Tc^r genes and chloride concentration, specific conductivity and temperature. The range of Spearman's rho calculated for the negative correlations for each of the sites along the stream network comparing abundance of Tc^r genes with chloride was $-0.870<rho< -0.788$; comparing abundance of Tc^r genes with specific conductivity was $-0.832<rho<-0.512$; and comparing abundance of Tc^r genes with temperature was $-0.885<rho<-0.524$. There were no statistically significant correlations between the average of 16S rRNA genes and any of the measured parameters ($-0.408 < rho < 0.395$) at the control site. Table 2 summarizes the calculated Spearman's rank correlations for the parameters for which data analyses of the individual sites revealed significant correlations at $p < 0.05$.

Table 2: Spearman rank correlation table for all sites along the Sumas River between total tetracycline resistance genes and total tetracycline resistance genes normalized to 16S rRNA genes compared with water quality and quantity parameters. Two-tailed significance is indicated in parentheses.

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	Instantaneous discharge	48 hour discharge	72 hour rainfall	Temp	Turbidity	Specific conductivity	Chloride	Total Tc^r genes	Tc^r genes normalized to 16S rRNA genes
Instantaneous discharge	1.000								
48 hour discharge	0.959	1.000							
72 hour rainfall	0.553 (0.026)	0.603 (0.013)	1.000						
Temperature	-0.648 (0.009)	-0.741 (0.002)	-0.39 (0.109)	1.000					
Turbidity	0.700 (0.004)	0.743 (0.004)	0.544 (0.020)	-0.520 (0.027)	1.000				
Specific conductivity	-0.579 (0.024)	-0.443 (0.098)	-0.430 (0.075)	No significant correlation	-0.493 (0.038)	1.000			
Chloride	-0.302 (0.029)	-0.609 (0.010)	No significant correlation	No significant correlation	-0.254 (0.032)	0.478 (0.053)	1.000		
Total Tc^r genes	0.621 (0.013)	0.661 (0.007)	0.482 (0.043)	-0.605 (0.008)	0.534 (0.023)	-0.613 (0.007)	-0.513 (0.035)	1.000	
Tc^r genes normalized to 16S rRNA genes	0.578 (0.030)	0.556 (0.039)	0.571 (0.017)	-0.631 (0.007)	0.611 (0.007)	-0.751	-0.683 (0.002)	0.924	1.000

3.3 Estimation of Mass Transport of Tetracycline Resistance Genes

The Table 3 summarizes the calculated mass flux of the total tetracycline resistance genes measured along a finite continuous segment of the Sumas River (approximately 12.6 km). Four sites were chosen (see Figure 1) at locations near to farm facilities or large crop fields. Site 2 was located within about 50 m from a dairy barn and site 4 was located near (about 100 m) to a poultry barn. Both site 3 and site 5 were located immediately adjacent to large hay fields. The highest mass flux between 1.67×10^{11} and 1.16×10^{12} copies/sec occurred in November 2005 during a period of high total rainfall and statistically significantly higher ($4.16 \times 10^{10} - 2.29 \times 10^{11}$ copies/m^3) abundance of the Tc^r genes.

Table 3: Calculated mass transport rates of the total tetracycline resistance genes at four stations along one segment of the Sumas River between July and December, 2005 (Site 5 is the furthest downstream). Gene abundance calculated from the average of three replicates. (* indicates significantly higher mass flux at $p < 0.05$ than that measured in other months.)

	Discharge rate (m^3/s)	Total Tc^r genes ($copies/m^3$)	Mass Flux (copies/s)	Sites
July	1.03	2.17×10^6	2.24×10^6	2
	1.07	2.38×10^7	2.55×10^7	3
	1.40	2.69×10^6	3.76×10^6	4
	1.17	4.76×10^5	5.56×10^5	5
August	2.00	1.09×10^6	2.18×10^6	2
	0.61	2.86×10^7	1.74×10^7	3
	0.62	1.11×10^6	6.90×10^5	4
	0.98	9.60×10^5	9.43×10^5	5
September	0.82	1.81×10^6	1.48×10^6	2
	1.10	1.19×10^7	1.31×10^7	3
	1.11	4.45×10^6	4.94×10^6	4
	0.89	2.28×10^7	2.04×10^7	5
November	4.08	2.29×10^{11}	9.34×10^{11}*	2
	5.38	2.16×10^{11}	1.16×10^{12}*	3
	4.56	6.32×10^{10}	2.88×10^{11}*	4
	4.00	4.16×10^{10}	1.67×10^{11}*	5
December	2.58	4.15×10^8	1.07×10^9	2
	2.89	1.47×10^8	4.24×10^8	3
	4.46	1.80×10^8	8.06×10^8	4
	2.34	1.54×10^7	3.59×10^7	5

4 DISCUSSION

Land use factors likely contributed to the observed higher abundances of Tc^r genes in the Sumas River stream network when compared with the control site (Figure 6). Agricultural land use along the stream reach in the immediate proximity of all sampling locations of this study included dairy operations, poultry and swine farms and large crops fields. Of 27 individual land parcels observed within 500 m of the stream network under study, 14 of these farming operations produced poultry or livestock while 9 other farms produced corn or hay. Popowska et al. (2012) detected *tet*(M), *tet*(O) and *tet*(W) by qPCR analyses in soils collected from farmland in the area of Lesznowola, Poland and demonstrated that more diverse populations of bacteria with resistance were present in the agricultural soil samples. Higher relative abundance of Tc^r genes (some in the order of 10^5 to 10^7 gene copies /mL) in soils collected near livestock farms in agricultural regions in China have been described (Wu et al., 2010; Ji et al., 2012; Zhu 2013). The water quality trends described in this study (with the exception of the Tc^r genes that had not previously been measured) had been observed for the same sampling sites (Berka et al., 2001) and differences in flow conditions for the Sumas stream network (often slower discharge rates) had been recorded (Shead, 2004). Italian researchers have demonstrated correlations between ARGs and water quality parameters, specifically nitrogen and phosphorus compounds and total organic carbon, in aquatic ecosystems following rainfall events (Di Cesare et al., 2015). Observations presented in this study suggest that agricultural land use activities likely contributed to the potential for runoff of ARGs into the stream network.

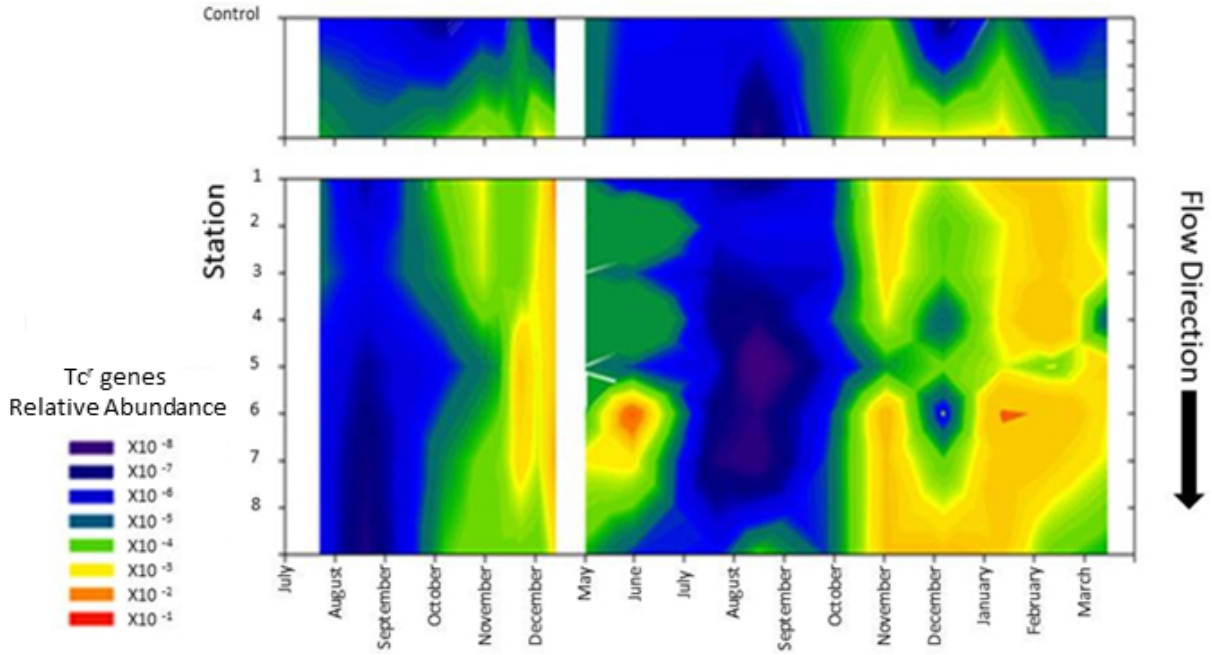

Figure 5: Seasonal and spatial trends in total tetracycline resistance genes normalized to 16S rRNA genes along all sites of the investigated segment of the Sumas River stream network for the period of July 2004 – March 2006.

The patterns of Tc^r abundances illustrated in Figure 5 likely reflected seasonal weather and land-use patterns. Previous research in the Sumas watershed has demonstrated that nutrient levels, fecal coliform counts, concentrations of suspended particulate matter and other environmental contaminants (including metals) increased during the wet season (Berka, 1996; MacDonald, 2005, Ross, 2006; Solano 2006). It is likely that rainfall influenced the runoff and soil erosion from agricultural fields that, in turn, contributed to the higher relative abundance of Tc^r genes observed during wet seasons in this study. Similar patterns of gene abundances and upstream agricultural activity have been observed (Pruden et al., 2012; Heuer et al., 2011; Luby et al., 2016; Nõlvak et al., 2016) and increased abundances occur regardless of antibiotic usage in animals (Udikovic-Kolic et al., 2014). Coupled with seasonal variation of stream flow (e.g.

Knapp et al., 2012), agricultural activities can dramatically alter the transport of ARGs through the aquatic receiving environment in the Sumas watershed. Positive Spearman's Rank correlation between turbidity and Tc^r genes and the seasonal increase in turbidity as rainfall events become more frequent and intense during the year suggests that Tc^r genes associated with particulates can be transported in receiving waters. The results of this study combined with evidence gathered from researchers in several regions of the world support the conclusion that soil, manure and water in agricultural locations are possible hotspots of antibiotic resistance genes and antibiotic resistant bacteria (Thanner et al., 2016) and that environmental factors influence abundance and transport of ARGs in agricultural watersheds.

As with most agricultural regions, land application of manure on fields is used for soil conditioning, crop fertilization and agricultural waste disposal in the Sumas watershed. Composted manure is distributed on fields using tractor-drawn equipment and liquid manure is spread through irrigation systems when soils are not excessively saturated. In the province of British Columbia, there are no explicit regulations that limit field application of manure between specific dates although most field fertilization occurs between April and October. The BC Ministry of Agriculture Code of Farm Practice (2014) states that spreading of manure "is not advised during periods of high rainfall or on snow-covered ground." Manure management practices conducted during various times of the year are likely to play a role in the introduction of contaminants to the stream network of the Sumas watershed during periods of higher rainfall.

Manure application on land is a likely contributor to elevated abundance of tetracycline resistance genes in bacteria collected from the Sumas River. Tetracycline resistance genes

conferring ribosomal protection (i.e. *tet* (W) and *tet* (Q)) were most abundant, which reflect profiles of genes from animal fecal samples (Patterson et al., 2007; Peak et al., 2007).

In this study, the abundances of the four Tc^r genes measured in the stream network varied with season, with significantly higher abundances observed during rainy months (October – January), and this is reflected in water quality patterns. This and previous research in the Sumas watershed (MacDonald, 2005, Ross, 2006; Solano 2006) demonstrated that seasonal variation in nitrate concentrations indicate agricultural non-point source pollution and elevated measurement levels occurred during the wet winter months, whereas lower nitrate values were recorded in summer low flow conditions (Berka et al., 2001; Schreier et al., 2001; Ross, 2006; Solano, 2006). Significantly higher orthophosphate concentrations were measured in the Sumas stream network during the wet season months than those in the control stream also suggesting that surface run-off fertilizers contributed to the contaminant load in receiving waters. Conversely, specific conductivity and measurements of chloride were higher (although not statistically significant) in the dry season months than during wet season months in the stream network, which reflects considerable contribution of groundwater to the stream at low surface flows (Berka, 1996; Solano, 2006) and dilution during periods of precipitation.

Rainfall events influenced the transport of Tc^r genes in surface waters. Relationships between the abundance of Tc^r genes and specific water quality parameters were observed at instantaneous discharge, 48 h discharge, 72 h rainfall suggesting that intensity and duration of rainfall events influenced the abundance of Tc^r genes in the flowing water. Manure-borne bacteria have been shown to be transported by rainfall or irrigation into surface waters via soil and organic particles

(Jeng et al., 2005; Pachepsky et al., 2008) and higher discharge rates related to stormwater runoff have been associated with higher turbidity in urban and rural streams (Mallin et al., 2009).

Transport of tetracycline resistance genes through the environment could be observed during wet fall and winter seasons and the gene flux estimates of the indicator Tc^r genes mirrored the stream discharge profiles and gene concentrations (Table 3). The location of dairy farms, poultry operations and large crop fields together with the timing of manure application on the adjacent land along the specific stream reach could have influenced observed seasonal Tc^r gene flux. The highest Tc^r gene mass flux was measured in November which correlates to a period immediately after the time when most manure was spread on land in the fall and the beginning of more intense winter rainfall. It is likely that local variation in Tc^r gene flux resulted from differences in inputs with space and time. Field fertilization with animal manure combined with seasonal precipitation patterns likely contributed to the Tc^r gene flux via soil erosion and surface runoff.

5 CONCLUSIONS

The presence of ARGs at elevated abundance in streams and transport of these genes through water courses could potentially provide opportunities for *de novo* induction of resistance in environmental bacteria. Changes in weather patterns, such as shorter duration high intensity rainfall events, increase the potential for soil erosion and elevate the concentration of particulate matter and associated contaminants in rivers and streams. This research confirms that increased abundance of some specific tetracycline resistance genes were present in the agricultural stream network of the Sumas watershed and has demonstrated that ARGs can be co-lineated with the suspended material in aquatic systems. Therefore higher intensity rainfall events are likely to increase both suspended particulates and ARGs in agricultural watersheds as a result of soil

erosion. Equally important, results of this case study demonstrate that during periods of considerably lower rainfall in warm summer months, the concentrations of particulate matter and bacteria were lower while the abundance of ARGs remained proportionately high. It is likely that agricultural activities and land use practises in the watershed contributed to elevated concentrations of nitrate, phosphate, chloride and tetracycline resistance genes that were measured. These observations underscore the importance of making mindful decisions in land use and management in agricultural watersheds and that minimizing risks linked to the spread of antibiotic resistance requires limiting the circulation of ARGs into and through the environment.

ACKNOWLEDGMENTS

The authors wish to thank Paula Parkinson, Susan Harper, and Raphaël Fugère for their assistance with laboratory analyses and fieldwork. Financial assistance for this research was supported by contributions from a Natural Science and Engineering Research Council (NSERC) grant to Dr. Ken Hall, from a U.S. Environmental Protection Agency STAR Grant to Dr. David Graham, and from the Canadian Water Network Centres of Excellence.

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