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Antibiotic resistance genes, class 1 integrons, and IncP-1/IncQ-1 plasmids in irrigation return flows☆

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ABSTRACT

Surface waters could be a dominant route by which antibiotic resistance genes (ARGs) are disseminated. In the present study we explored the prevalence and abundance of ARGs [$bla_{CTX\text{-}M\text{-}1}$, *erm*(B), *sul1*, *tet*(B), *tet*(M), and *tet*(X)], class 1 integron-integrase gene (*intI1*), and IncP-1 and IncQ-1 plasmids in eight irrigation return flows (IRFs) and a background site (Main Line Canal, MLC) in the Upper Snake Rock watershed in southern Idaho. Grab samples were collected on a monthly basis for a calendar year, which were processed to extract microbial DNA, followed by droplet digital PCR to quantify the gene copies on an absolute (per 100 mL) and relative (per 16S rRNA gene copies) basis. The antibiotic resistance and *intI1* genes and IncP-1/IncQ-1 plasmids were recovered at all IRF sampling sites with detections ranging from 55 to 81 out of 81 water sampling events. The $bla_{CTX\text{-}M\text{-}1}$ gene was detected the least frequently (68%), while the other genes were detected more frequently (88–100%). All of the genes were also detected at MLC from April to Oct when water was present in the canal. The genes from lowest to greatest relative abundance in the IRFs were: $bla_{CTX\text{-}M\text{-}1} < erm(B) < tet(B) <$ IncQ-1 $< tet(M) < sul1 < intI1 =$ IncP-1 $< tet(X)$. When compared to the average annual relative gene abundances in MLC water samples, they were found to be at statistically greater levels ($P \leq 0.008$) except that of the IncP-1 and IncQ-1 plasmids ($P = 0.8$ and 0.08, respectively). The fact that most IRFs contained higher levels than found in the canal water, indicates that IRFs can be a point source of ARGs that ultimately discharge into surface waters.

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1. Introduction

In arid regions of the United States, irrigation is necessary as precipitation during the growing season cannot support crop production. While the majority of irrigation waters are supplied by on-farm sources, about 25% of the irrigated land receives water from off-farm sources (USDA-NASS, 2019). In the latter case, irrigation projects deliver surface water to fields through canals, laterals, and ditches. Because the diversions cannot exactly match the irrigation demand for very large projects, excess water is diverted to ensure that irrigation supply meets the irrigation demand. The excess irrigation water becomes what is known as "irrigation return flow" (IRF) when it is not used within the watershed, which is then ultimately returned to a water body (Bjorneberg et al., 2015). Surface runoff and subsurface drainage also contribute to the IRF, with rainfall rarely contributing runoff in arid areas.

The water quality of a watershed, especially in those that contain large land areas under agricultural production, can be impacted by a variety of contaminants such as nutrients, pesticides, metals, sediments, and pathogens. While IRFs are point sources, they are exempt from discharge permit requirements under the Federal Water Pollution Control Act (1972). Regardless, IRFs are responsible for transferring contaminants from diffuse (nonpoint) sources to regulated surface water bodies such as streams and rivers (Bjorneberg et al., 2015; Stephenson and Street, 1978). In the U.S., over half of the streams and rivers that have been assessed are listed as having impaired water quality, with microbial pollution listed as the number one cause of impairment, followed by sediment and nutrients (U.S. EPA, 2019). The contamination of water resources with fecal material from humans and animals is a public and environmental health issue because associated pathogens can cause infection, illness, and sometimes death (Pandey, 2014). In addition to pathogens, their development of antibiotic resistance jeopardizes the future effectiveness of drugs used to treat life-threatening infections. Antibiotic resistance is encoded by antibiotic resistance genes (ARGs) and surface waters could be a dominant route by which they are disseminated (Pruden et al.,

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2012). There is abundant evidence that anthropogenic activities are responsible for altering the distribution and abundance of ARGs in various environmental compartments including surface water, soil, and sediment.

The Upper Snake Rock (USR) watershed in southern Idaho is an ideal system to investigate the pervasiveness of ARGs and other associated genetic elements in surface waters. The presence of surface water in the high-desert watershed is primarily related to irrigated agricultural production. As a result, it contains an extensive network of IRFs that ultimately discharge into the Snake River. The IRFs were determined to contain trace quantities of antibiotic residues as reported by Dungan et al. (2017). In the present study we investigated the prevalence and abundance of ARGs [$bla_{CTX\text{-}M\text{-}1}$, *erm*(B), *sul1*, *tet*(B), *tet*(M), and *tet*(X)], class 1 integron-integrase gene (*intI1*), and IncP-1 and IncQ-1 plasmids in eight IRFs in the watershed on a monthly basis during a calendar year. The ARGs were selected because they include resistance to antibiotics that are considered medically important (WHO, 2018), while *intI1* was targeted since it is linked to genes conferring resistance to antibiotics and has been determined to be a good proxy for anthropogenic pollution (Gillings et al., 2015). Plasmids of the incompatibility group IncP-1 and IncQ-1 were targeted because they contain antibiotic resistance traits and are assumed to facilitate horizontal gene transfer due to their efficient conjugative transfer to and stable replication in a broad range of hosts (Jechalke et al., 2013; Loftie-Eaton and Rawlings, 2012; Popowska and Krawczyk-Balska, 2013). In addition to gene surveillance in the IRFs, water quality characteristics were determined and correlated with the genes. Correlations among the genes were also performed with a specific interest in determining if the IncP-1 and IncQ-1 plasmids influence the accumulation of ARGs in the surface waters.

2. Materials and methods

2.1. Watershed sampling sites

Since 2005, the Twin Falls tract of the USR watershed has been part of the USDA Conservation Effects Assessment Project (CEAP) whose purpose is to determine the effectiveness of conservation practices in an irrigated watershed (Bjorneberg et al., 2008). The Twin Falls tract is 820 km^2 and is located along the south side of the Snake River (Fig. 1). Water samples were collected from eight IRF sampling sites: Cedar Draw (CD), Deep Creek (DC), Hansen Coulee (HC), I Coulee (IC), Mud Creek (MC), N Coulee (NC), Rock Creek Poleline (RCP), and Twin Falls Coulee (TFC). A sample was also collected from the Main Line Canal (MLC) which supplies more than 75% of the water to the Twin Falls tract (Bjorneberg et al., In press). The source of water in the canal system is Snake River water, thus the MLC sample was considered to provide background data for the present study.

2.2. Water sample collection

Biweekly water samples were collected from 9 Jan to 18 Dec 2018. Samples were collected from DC, MC, IC, CD, and RCP from Jan to early April since these streams flow all year due to subsurface drainage. Once the irrigation season began in mid April, water could also be collected from NC, TFC, HC, and MLC, with the last set of samples collected from these sites on 23 Oct. One water sample was collected at each site. Water samples were placed in sterile plastic bottles, then stored in a cooler until brought to the laboratory. At the laboratory the water samples were immediately placed under refrigeration at 5 °C. All samples were processed within 24 h of collection. Average water quality values are presented in Table 1.

2.3. DNA extraction

To extract DNA from the water samples, they were first passed through a Sterivex™ 0.22 μm pore-size hydrophilic polyvinylidene fluoride membrane (Cat no. SVGVL10RC; Millipore Sigma, Burlington, MA, USA). Depending upon the suspended sediment level, the volume filtered ranged from 120 to 1740 mL. The filters were then processed using the DNeasy® PowerWater Sterivex kit (Qiagen, Inc., Hilden, Germany) according to the manufacturer's

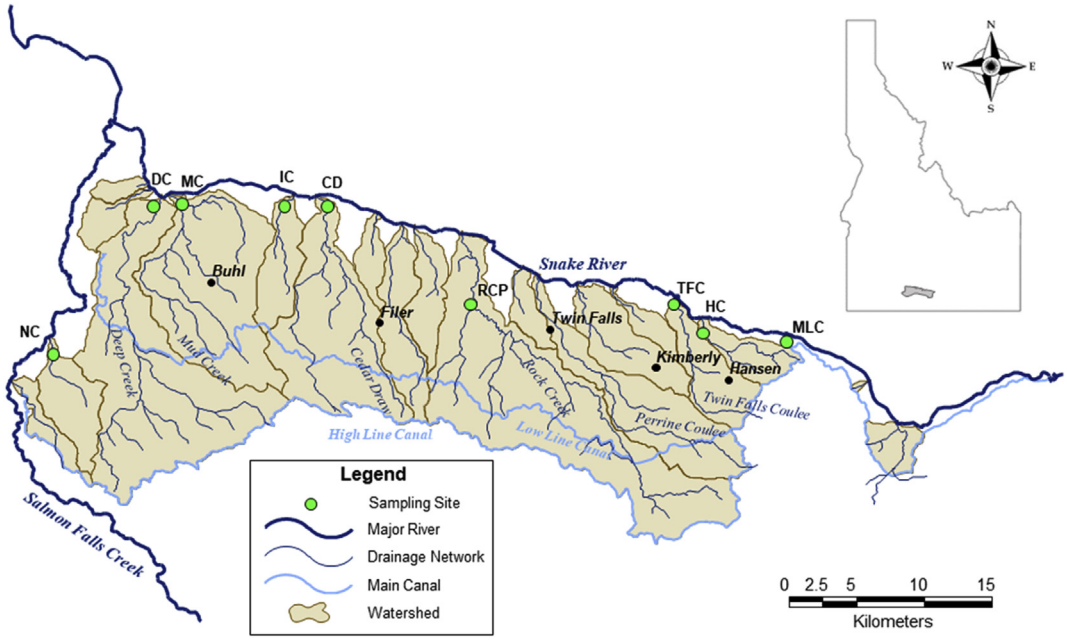

Fig. 1. The water sampling sites within the Upper Snake Rock watershed in south-central Idaho. The irrigation return flow sites from west to east were N Coulee (NC), Deep Creek (DC), Mud Creek (MC), I Coulee (IC), Cedar Draw (CD), Rock Creek Poleline (RCP), Twin Falls Coulee (TFC), and Hansen Coulee (HC), while the background site was the Main Line Canal (MLC).

Table 1
Average water quality values for the irrigation return flows and background canal site (MLC).

Site	Water Temp °C	pH	EC μS cm^{-1}	TSS mg L^{-1}	Diss NH_4^+ mg L^{-1}	Diss NO_3^- mg L^{-1}	Diss P mg L^{-1}	Total N mg L^{-1}	Total P mg L^{-1}
NC	16.9 (5.9[a])	8.3 (0.1)	386 (23)	89.4 (68.1)	0.11 (0.06)	0.04 (0.12)	0.05 (0.06)	0.72 (0.20)	0.17 (0.09)
DC	13.7 (5.3)	8.4 (0.1)	679 (184)	45.8 (43.9)	0.09 (0.04)	3.4 (2.7)	0.01 (0.02)	0.57 (0.18)	0.06 (0.05)
MC	13.9 (4.3)	8.3 (0.1)	809 (93)	26.8 (18.4)	0.14 (0.05)	3.5 (1.4)	0.04 (0.02)	0.55 (0.20)	0.07 (0.04)
IC	12.8 (5.4)	8.3 (0.2)	716 (112)	96.6 (109)	0.10 (0.05)	3.6 (2.1)	0.01 (0.02)	0.55 (0.24)	0.09 (0.10)
CD	12.5 (5.3)	8.3 (0.1)	688 (186)	43.5 (31.6)	0.07 (0.05)	2.7 (2.0)	0.03 (0.03)	0.51 (0.21)	0.07 (0.04)
RCP	12.1 (3.7)	8.2 (0.2)	699 (101)	33.3 (22.7)	0.11 (0.04)	2.6 (1.0)	0.01 (0.02)	0.54 (0.59)	0.05 (0.05)
TFC	16.1 (5.5)	8.3 (0.1)	384 (25)	104.2 (96.1)	0.11 (0.04)	0.002 (0.008)	0.00 (0.01)	0.52 (0.16)	0.09 (0.06)
HC	15.8 (4.3)	8.0 (0.3)	403 (35)	38.0 (29.1)	0.12 (0.07)	0.04 (0.12)	0.03 (0.03)	0.53 (0.19)	0.09 (0.06)
MLC	17.8 (5.3)	8.6 (0.1)	371 (31)	29.2 (11.9)	0.10 (0.05)	0.004 (0.014)	0.002 (0.004)	0.52 (0.15)	0.03 (0.02)

[a] Values in parentheses are standard deviation (n = 13 [HC, MLC], 14 [NC, TFC], or 24 [CD, DC, IC, MC, RCP]).

protocol. One DNA extraction was performed per sample. The concentration and quality of the extracted DNA was determined using a NanoDrop™ 2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). The DNA was stored in DNase-free water at −75 °C until droplet digital PCR (ddPCR) was performed.

2.4. Gene quantification

Droplet digital PCR was performed using the Bio-Rad QX200™ system (Hercules, CA, USA). In brief, 10-fold dilutions of the DNA extracts were utilized as template in PCR reaction mixtures using primer and probe sets listed in Table 2. Each mixture contained 12.5 μL of 2 × ddPCR supermix for probes (no dUTP; Bio-Rad), 900 nM of forward and reverse primers (except IncP1-Fz, IncP1-Rge, and IncP1-Rd at 450 nM), 250 nM of probe, 2 μL of diluted DNA template, and sterile DNase-free water to a final volume of 25 μL. Probes include a 6-FAM 5′ fluorophore, ZEN internal quencher, and 3′ Iowa Black FQ quencher (Integrated DNA Technologies, Skokie, IL). Using the QX200 droplet generator with DG8™ cartridge and gasket (Bio-Rad), each reaction mixture was processed with droplet generation oil for probes (Bio-Rad) to produce approximately 20,000 droplets. Afterwards, 40 μL of droplets were transferred using an 8-channel pipette into a 96-well plate and heat sealed with pierceable foil. The plate was then placed into a T100™ thermal cycler and subjected to the following temperature conditions: 1 cycle at 95 °C for 3 min, then 40 cycles at 95 °C for 15 s and annealing temperature for 30 s. Upon completion, the 96-well plate was transferred to the QX200 droplet reader (Bio-Rad) and the droplets were scanned. The ddPCR data were analyzed using QuantaSoft™ Analysis Pro software (Version 1, Bio-Rad) to determine the concentration of the gene targets.

Table 2
Primer and probe sequences and annealing conditions used in the study.

Gene	Primer/Probe	Sequence (5′ to 3′)	T_A (°C)	Amplicon Size (bp)	Reference
bla$_{CTX-M-1}$	CTX-M-F	ATGTGCAGYACCAGTAARGTKATGGC	58	336	Birkett et al. (2007)
	CTX-M-R	ATCACKCGGRTCGCCNGGRAT			
	CTX-M-1-P	CCCGACAGCTGGGAGACGAAACGT			
erm(B)	ermB-F	GGATTCTACAAGCGTACCTTGGA	60	92	Bockelmann et al. (2009)
	ermB-R	GCTGGCAGCTTAAGCAATTGCT			
	ermB-P	CACTAGGGTTGCTCTTGCACACTCAAGTC			
sul1	sul1-F	CCGTTGGCCTTCCTGTAAAG	60	67	Heuer and Smalla (2007)
	sul1-R	TTGCCGATCGCGTGAAGT			
	sul1-P	CAGCGAGCCTTGCGGCGG			
tet(B)	tetB-F	ACACTCAGTATTCCAAGCCTTTG	60	205	Peak et al. (2007)
	tetB-R	GATAGACATCACTCCCTGTAATGC			
	tetB-P	AAAGCGATCCCACCACCAGCCAAT			
tet(M)	tetM-F	GGTTTCTCTTGGATACTTAAATCAATCR	60	88	Peak et al. (2007)
	tetM-R	CCAACCATAYAATCCTTGTTCRC			
	tetM-P	ATGCAGTTATGGARGGGATACGCTATGGY			
tet(X)	tetX-F	GCAAGCGCCCATTACCCATAA	60	97	McKinney et al. (2018)
	tetX-R	AAGGCATCCACCAACCCACT			
	tetX-P	CATTTGATGCCGCCTTTTGCAGGGC			
intI1	intI1-F	GCCTTGATGTTACCCGAGAG	60	196	Barraud et al. (2010)
	intI1-R	GATCGGTCGAATGCGTGT			
	intI1-P	ATTCCTGGCCGTGGTTCTGGGTTTT			
IncP-1	IncP1-F	TCATCGACAACGACTACAACG	60	118	Jechalke et al. (2013)
	IncP1-R	TTCTTCTTGCCCTTCGCCAG			
	IncP1-Fz	TCGTGGATAACGACTACAACG			
	IncP1-Rge	TTYTTCYTGCCCTTGGCCAG			
	IncP1-Rd	TTCTTGACTCCCTTCGCCAG			
	IncP1-P	TCAGYTCRTTGCGYTGCAGGTTCTCVAT			
	IncP1-Pgz	TSAGGTCGTTGCGTTGCAGGTTYTCAAT			
IncQ-1	IncQ1-F	CGARGAAYTATCAGGCAT	56	220	Thibault Stalder, Unpublished
	IncQ1-R	GTCTTGCCSYTGGAYTCM			
	IncQ1-P	CTTGTCCTTGCGGTTGGT			
16S rRNA	BACT1369F	CGGTGAATACGTTCYCGG	56	~123	Suzuki et al. (2000)
	PROK1492R	GGWTACCTTGTTACGACTT			
	TM1389F-P	CTTGTACACACCGCCCGTC			

2.5. Statistical analysis

Relative gene abundance data (gene copies[16S rRNA gene copies]$^{-1}$) was $\log_{10}$ transformed before analysis. Pearson correlation coefficients (r) were calculated using the CORR procedure of SAS 9.4 (SAS Institute Inc., Cary, NC), with highly correlated values defined as $r \geq 0.70$. To compare the relative gene abundances between the IRF and MLC sites, one-way analysis of variance was performed using the ANOVA procedure with comparisons determined with Dunnett's t-test (SAS Institute Inc., Cary, NC). Statements of statistical significance were declared at $P < 0.05$.

3. Results and discussion

Watershed IRFs provide a unique opportunity to monitor the influence of land use practices on the occurrence and abundance of ARGs and other related genetic determinants, as well as assess which genes will ultimately be transferred to riverways. In the present study, antibiotic resistance and *intI1* genes and IncP-1/IncQ-1 plasmids were recovered at all IRF sampling sites with detections in 55–81 of the 81 water sampling events (Fig. 2). The $bla_{\text{CTX-M-1}}$ gene was detected the least frequently (68%), while the other genes were detected more frequently (88–100%). Their absolute abundances ranged from 6.1E+00 to 2.9E+05 gene copies (100 mL)$^{-1}$ and average levels for each gene were: $bla_{\text{CTX-M-1}}$, 8E+01; *erm*(B), 1.1E+02; *sul1*, 1.5E+04; *tet*(B), 2.8E+02; *tet*(M), 8.6e+03; *tet*(X), 7.3E+03; *intI1*, 2.3E+04; IncP-1, 2.2E+04; and IncQ-1, 8.0E+02 (Fig. 2). Of the ARGs targeted, it was unexpected that we would detect $bla_{\text{CTX-M-1}}$, as we have not detected it in three soil-focused studies of ours in this region of Idaho (Dungan et al., 2018; Dungan et al., 2019; McKinney et al., 2018). While CTX-M enzymes are the most prevalent extended-spectrum β-lactamases (ESBLs), there have only been sporadic reports of CTX-M-producing isolates in the U.S. (Canton and Coque, 2006). Our ability to detect $bla_{\text{CTX-M-1}}$ in the present study was likely related to fact that we utilized ddPCR, which is a more sensitive technique compared to quantitative real-time PCR (Hindson et al., 2011; Rothrock et al., 2013). All of the genes were also detected at the background site (MLC) from April to Oct when water was present in the canal. At MLC, the average gene copies (100 mL)$^{-1}$ were 1.7- to 6.2-fold lower than at the IRF sites, except in the case of IncP-1, which was 1.5-fold greater (data not shown). The average 16S rRNA gene levels at the IRF and MLC sites were 1.2E+08 and 2.5E+08 copies (100 mL)$^{-1}$, respectively. At the IRF sites, the absolute abundance of the 16S rRNA gene was lowest from Jan to Mar, then increased in April and remained ≥1.0E+08 gene copies (100 mL)$^{-1}$ until Oct (Fig. 3). At MLC, which was only sampled from April to Oct, the absolute 16S rRNA gene levels ranged from 1.8E+08 to 6.4E+08 gene copies (100 mL)$^{-1}$ (data not shown). The 16S rRNA gene can be used as a general indicator of the total bacterial population size and these data indicate that it was highest during the irrigation season (i.e., April to Oct). One would expect a larger bacterial population during these months because of increased inputs from surface irrigation runoff and higher water temperatures that generally support abundant microbial growth.

The ARGs, *intI1* gene and IncP-1/IncQ-1 plasmids were normalized to the 16S rRNA gene to account for differences in the bacterial population, with average annual results presented in Fig. 4. The genes from lowest to greatest relative abundance in the IRFs were: $bla_{\text{CTX-M-1}} < erm(\text{B}) < tet(\text{B}) <$ IncQ-1 $< tet(\text{M}) <$ *sul1* $<$ *intI1* $=$ IncP-1 $< tet(\text{X})$. When compared to the average annual relative gene abundances in MLC water samples, they were found to be at statistically greater levels ($P \leq 0.008$) except that of the IncP-1 and IncQ-1 plasmids ($P = 0.8$ and 0.08, respectively). The average relative gene abundance over the course of the study with the IRF site data combined and compared to that of the MLC site is presented in Fig. 5. In the IRFs, the relative abundance of all gene targets was generally higher during the winter, lowest in April, but then gradually increased throughout the remainder of the year. To provide more detail, the relative gene abundances by sampling site have been provided in Table 3. A one-way analysis of variance was conducted on this data to compare the gene abundance in the IRFs sites against those in MLC. It was found in many instances that the average relative abundances of the antibiotic resistance and *intI1* genes were greater in the DC, MC, IC, CD, and RCP return flows, with levels ranging from 9.3E-07 to 8.1E-04 gene copies (16S rRNA gene copies)$^{-1}$. This is an interesting observation because these particular IRFs receive outflow from subsurface drain tunnels, which were installed horizontally in the bedrock to eliminate localized high water tables that formed as a result of irrigation (Bjorneberg et al., 2008; Lentz et al., 2018). As a result of this enhanced water

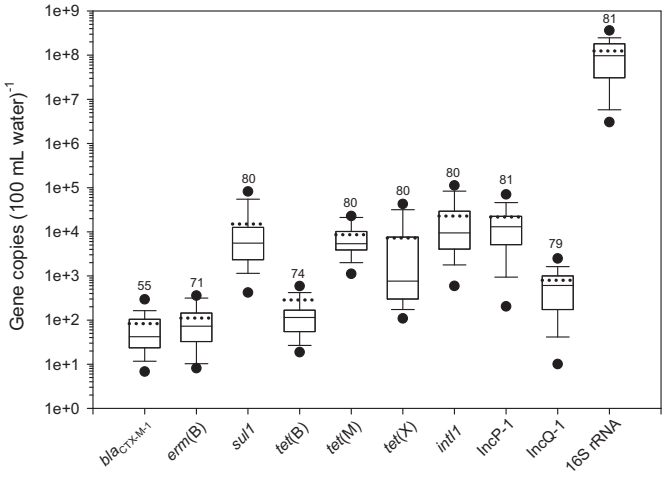

Fig. 2. The absolute abundance (gene copies/100 mL water) of antibiotic resistance genes [$bla_{\text{CTX-M-1}}$, *erm*(B), *sul1*, *tet*(B), *tet*(M) and *tet*(X)], class 1 integron-integrase gene (*intI1*), IncP-1 and IncQ-1 plasmids, and 16S rRNA gene in irrigation returns flows in the Upper Snake Rock watershed in south-central Idaho. The black horizontal lines in the box plots, from bottom to top including the whisker caps, represent the 10th, 25th, 50th, 75th and 90th percentiles, while the black dotted line is the mean and black circles are the 5th and 95th percentiles. The values above the box plots are the number of gene detections out of 81 sampling events.

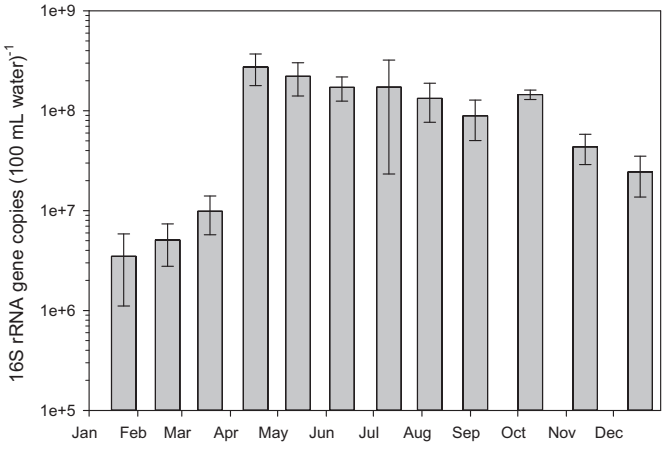

Fig. 3. The average absolute abundance (gene copies/100 mL water) of the 16S rRNA gene in the irrigation return flows for each month from Jan to Dec 2018. Error bars represent 95% confidence intervals.

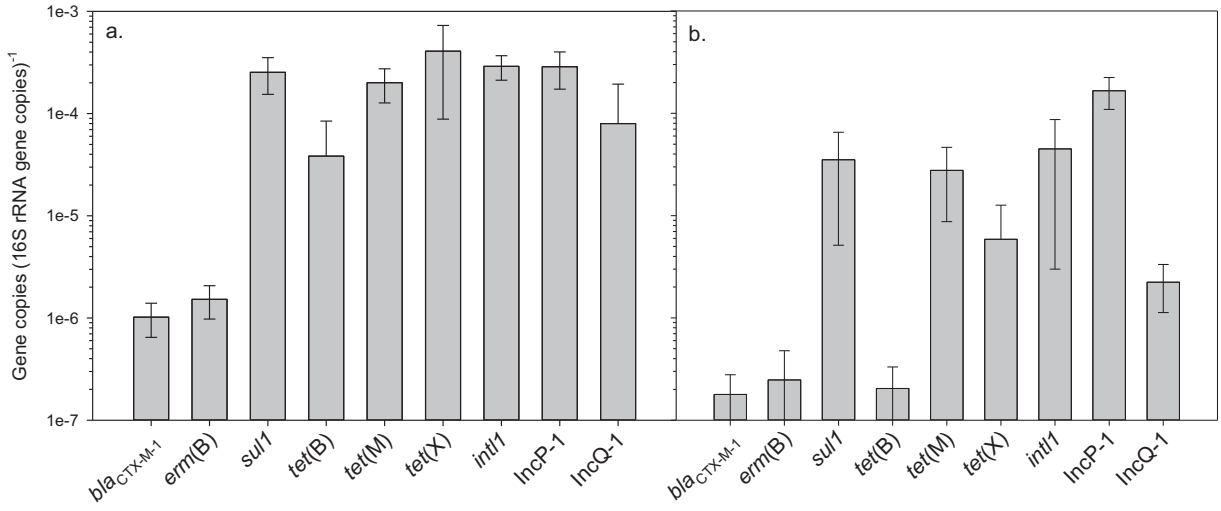

Fig. 4. The average annual relative abundance (gene copies/16S rRNA gene copies) of the antibiotic resistance genes [$bla_{CTX\text{-}M\text{-}1}$, *erm*(B), *sul1*, *tet*(B), *tet*(M) and *tet*(X)], class 1 integron-integrase gene (*intI1*), and IncP-1 and IncQ-1 plasmids in the a) irrigation return flows and b) background site (MLC). Error bars represent 95% confidence intervals.

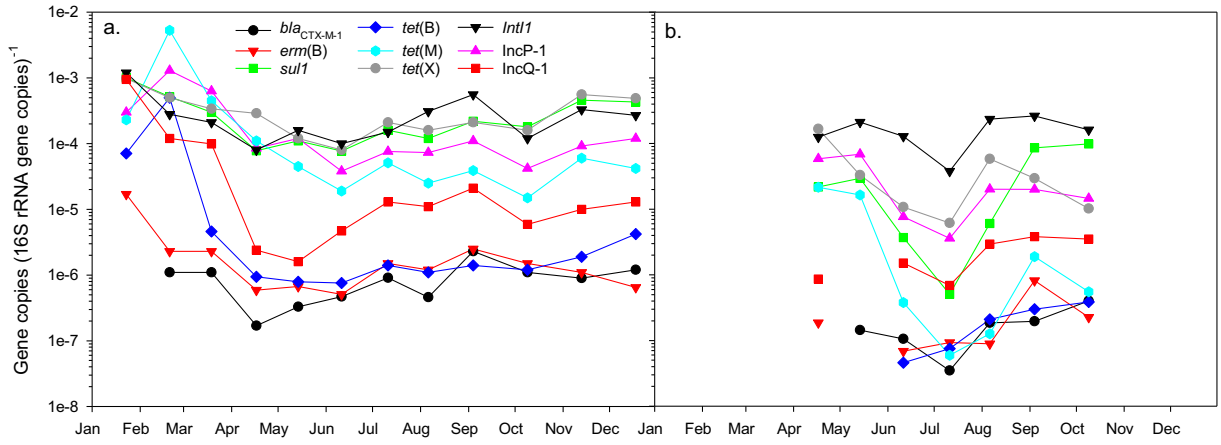

Fig. 5. The average relative abundance (gene copies/16S rRNA gene copies) of the antibiotic resistance genes [$bla_{CTX\text{-}M\text{-}1}$, *erm*(B), *sul1*, *tet*(B), *tet*(M) and *tet*(X)], class 1 integron-integrase gene (*intI1*), and IncP-1 and IncQ-1 plasmids for each month from Jan to Dec 2018 in the a) irrigation return flows and b) background site (MLC).

movement, salt and nitrate concentrations are greater in IRFs with subsurface drainage and it explains why the levels are higher in these particular IRFs (Table 1). It appears that some facet of the drain tunnels is also causing the antibiotic resistance and *intI1* genes to occur at higher levels than found in MLC, whereas this was generally not the case in IRFs that do not receive drain tunnel outflow (i.e., NC, TFC, and HC). This could potentially be the result of drain tunnels transporting a higher load of ARGs to the IRFs and/or nitrate enriching bacteria with ARGs. Of all IRF sampling sites, NC was the only location where none of the genes were found at a greater abundance than in MLC. This likely occurred since the NC return flow passes through a very small portion of the watershed on the far western edge and it does not receive surface/subsurface drainage from a large area like many of the other IRFs. Regarding plasmids, only the IncQ-1 plasmid in CD was found to be at a statistically greater relative abundance than in MLC.

The detection of the ARG targets in this study was not unexpected, as all of the genes (except $bla_{CTX\text{-}M\text{-}1}$) have been detected in agricultural and non-agricultural soils in this watershed (Dungan et al., 2019), as well as in dairy wastewater and manure-amended soils (Dungan et al., 2018; McKinney et al., 2018). However, because these genes were detected in total DNA extracts, it is not known if they were present in live or dead cells or extracellular; nevertheless, these data provide a good indication that agricultural land-use practices and possibly other anthropogenic sources (e.g., septic systems, wastewater plant effluents) are influencing the level of ARGs in IRFs. Bacteria can acquire resistance when they obtain ARGs through conjugation (cell-to-cell), transformation (uptake of naked DNA), or transduction (phage mediated). Both transformation and transduction are generally believed to have a limited influence on the dissemination of ARGs (von Wintersdorff et al., 2016). Regardless of the gene acquisition mechanism, little is known about the rate at which ARGs are transferred within environmental settings (Ashbolt et al., 2013; Durso and Cook, 2014). Despite knowledge gaps, there is evidence that clinically relevant ARGs have originated from environmental microbes (Wright, 2010), thus monitoring the occurrence and persistence of environmental ARGs is important, as it can provide data that can potentially help inform mitigation strategies (Fahrenfeld et al., 2014). It is important to reiterate that the ARGs, *intI1* gene, and IncP-1/IncQ-1 plasmids were all detected in MLC, which consists of water from the Snake River and is not considered to be pristine, as it receives agricultural, municipal, and industrial inputs prior to its diversion into the canal system. While no studies examining antibiotic resistance in IRFs

Table 3
Summary of relative gene abundances (gene copies/16S rRNA copies) in the irrigation return flows and background site (MLC) in the Twin Falls tract of the Upper Snake Rock watershed.

Site		$bla_{CTX-M-1}$	*erm*(B)	*sul1*	*tet*(B)	*tet*(M)	*tet*(X)	*intl1*	IncP-1	IncQ-1
NC	Min	6.5E-08	2.0E-07	2.0E-06	1.8E-07	8.8E-06	3.5E-07	2.3E-05	5.7E-05	1.5E-07
	Max	9.0E-07	2.7E-06	8.9E-05	6.0E-07	1.3E-04	1.8E-04	3.0E-04	1.6E-03	1.4E-05
	Mean	5.8E-07	7.2E-07	3.4E-05	3.6E-07	4.9E-05	3.2E-05	1.2E-04	4.4E-04	4.5E-06
	n = 7	3[a]	7	7	6	7	7	7	7	7
DC	Min	9.8E-08	2.4E-07	2.7E-05	3.8E-07	2.4E-05	2.1E-06	6.9E-05	3.5E-05	6.9E-07
	Max	2.0E-06	3.2E-06	3.0E-04	5.0E-04	1.6E-03	6.1E-03	3.3E-04	2.8E-04	2.8E-05
	Mean	6.5E-07	**1.1E-06**[b]	**1.0E-04**	5.6E-05	**3.0E-04**	**5.7E-04**	**1.6E-04**	1.5E-04	9.1E-06
	n = 12	9	11	12	9	12	12	12	12	12
MC	Min	3.8E-07	2.2E-07	3.4E-04	5.0E-07	7.8E-05	9.6E-05	3.2E-04	4.5E-05	1.0E-06
	Max	2.7E-06	2.7E-06	2.3E-03	8.6E-05	9.3E-04	1.0E-03	1.8E-03	1.7E-04	2.5E-05
	Mean	**9.4E-07**	8.7E-07	**1.1E-03**	**1.3E-05**	**3.0E-04**	**3.5E-04**	**8.5E-04**	1.2E-04	1.2E-05
	n = 12	8	9	12	12	12	12	12	12	12
IC	Min	3.8E-07	2.1E-07	1.2E-05	3.5E-07	2.1E-05	1.5E-06	2.6E-05	4.8E-05	3.3E-07
	Max	3.1E-06	7.1E-06	1.4E-04	9.9E-05	1.6E-03	4.0E-03	2.7E-04	3.8E-03	4.5E-03
	Mean	**1.6E-06**	**2.0E-06**	5.2E-05	**1.1E-05**	**2.6E-04**	**4.2E-04**	**1.1E-04**	4.5E-04	4.6E-04
	n = 12	7	10	11	11	11	11	11	12	10
CD	Min	1.1E-07	4.3E-07	3.1E-05	5.7E-08	2.5E-05	2.7E-06	4.7E-05	7.5E-05	4.4E-06
	Max	1.5E-06	3.0E-06	7.1E-04	1.7E-03	1.1E-03	9.4E-03	1.1E-03	1.7E-03	4.3E-04
	Mean	6.5E-07	**1.6E-06**	**2.1E-04**	**1.5E-04**	**2.0E-04**	**8.1E-04**	**4.3E-04**	4.5E-04	**9.3E-05**
	n = 12	9	10	12	12	12	12	12	12	12
RCP	Min	5.7E-08	1.6E-07	2.3E-05	2.1E-07	1.5E-05	1.3E-06	4.0E-05	4.8E-05	3.5E-07
	Max	9.8E-07	1.7E-05	8.3E-04	2.2E-04	1.1E-03	6.1E-03	9.5E-04	1.1E-03	1.9E-05
	Mean	5.5E-07	**2.4E-06**	**1.3E-04**	2.5E-05	1.6E-04	**5.4E-04**	**2.1E-04**	2.5E-04	5.8E-06
	n = 12	8	11	12	12	12	12	12	12	12
TFC	Min	2.1E-07	5.0E-07	1.1E-05	1.2E-07	2.5E-05	1.1E-06	1.3E-05	5.4E-05	2.0E-07
	Max	3.6E-06	7.6E-06	3.7E-04	2.8E-06	2.8E-04	1.4E-05	3.8E-04	1.6E-03	5.4E-05
	Mean	**1.6E-06**	**1.9E-06**	8.1E-05	1.1E-06	9.0E-05	7.4E-06	1.2E-04	3.3E-04	1.1E-05
	n = 7	4	7	7	5	7	7	7	7	7
HC	Min	1.2E-07	1.9E-07	5.1E-06	1.8E-07	3.2E-05	7.5E-07	1.4E-05	3.2E-05	4.2E-07
	Max	9.6E-06	4.9E-06	7.2E-05	3.3E-06	1.8E-04	3.4E-04	2.8E-04	3.3E-04	1.5E-05
	Mean	**1.9E-06**	1.3E-06	3.0E-05	1.1E-06	9.2E-05	5.6E-05	7.1E-05	1.2E-04	7.7E-06
	n = 7	7	6	7	7	7	7	7	7	6
MLC	Min	3.5E-08	6.9E-08	5.1E-07	4.6E-08	3.6E-06	5.9E-08	6.2E-06	3.8E-05	6.9E-07
	Max	4.0E-07	8.2E-07	9.9E-05	3.9E-07	6.9E-05	2.2E-05	1.7E-04	2.6E-04	3.8E-06
	Mean	1.8E-07	2.5E-07	3.5E-05	2.0E-07	2.8E-05	5.9E-06	4.5E-05	1.7E-04	2.2E-06
	n = 7	6	6	7	5	7	7	7	7	6

[a] Number of water samples with gene detections.
[b] Bold mean values indicate a significant difference at the 0.05 probablity level from that of MLC.

have been conducted to our knowledge, in riverine studies, a strong relationship has been found between ARG magnitude and livestock operations and wastewater treatment plant sources (Pruden et al., 2012). In water samples from the Sumas River of British Columbia, tetracycline resistance genes were found to be more abundant during wet months in an agricultural region than in a forested headwater control site (Keen et al., 2018). Pruden et al. (2006) detected a few tetracycline and sulfonamide resistance genes in sediment from pristine sections of the Poudre River in northern Colorado, but the genes were detected more frequently and at greater levels in downstream areas impacted by heavy agricultural and urban activity. Because ARGs are found in natural environments not under direct human influence (Allen et al., 2009; D'Costa et al., 2011), it is highly warranted to take into account background gene levels in affected sites to ensure impacts are properly evaluated in the context of naturally occurring levels of resistance (Durso et al., 2016; Rothrock et al., 2016).

Class 1 integrons contain one or more gene cassettes that encode antibiotic resistance and are often located on mobile genetic elements (MGEs), thus the integrase gene (*IntI1*) of class 1 integrons was tracked as an indicator of horizontal gene transfer potential. Correlation analyses were performed between the relative abundance of *intI1* and the ARGs (Table 4). Although statistically significant correlations were found with *erm*(B) and all three *tet* genes, the strongest correlation was between *intI1* and *sul1* ($r = 0.86$, $P < 0.001$), while all other correlations with *intI1* had $r \leq 0.66$. A strong correlation between *intI1* and *sul1* is often

Table 4
Pearson correlation analysis between the relative abundance (gene copies/16S rRNA gene copies) of the antibiotic resistance genes and mobile genetic elements.

	intl1	IncP-1	IncQ-1
$bla_{CTX-M-1}$	0.22	0.33 *	0.47 ***
erm(B)	0.28 *	0.29 *	0.41 ***
sul1	0.86 ***	0.27 *	0.45 ***
tet(B)	0.51 ***	0.21	0.49 ***
tet(M)	0.57 ***	0.13	0.33**
tet(X)	0.66 ***	0.01	0.15

*$P < 0.05$
**$P < 0.01$
***$P < 0.0001$

reported in environmental studies (Dungan et al., 2018; Lin et al., 2016; Nõlvak et al., 2016; Su et al., 2012) and is expected since *sul1* is a typical component of class 1 integrons (Mazel, 2006). Class 1 integrons can be located on IncP-1 plasmids, which may contribute to the accumulation and spread of resistance in the environment (Heuer et al., 2012). There were no strong correlations ($r \leq 0.33$) between the relative abundances of IncP-1 and ARGs, suggesting that IncP-1 was not likely responsible for the elevated ARG levels in the IRFs. IncQ-1 plasmids are of great interest because their accessory genes are dominated by ARGs, they are capable of replication in a very broad host range, and are highly mobilizable (Loftie-Eaton and Rawlings, 2012). Similar to IncP-1, no strong correlations were found between IncQ-1 and ARGs ($r \leq 0.49$),

although many of the relationships were significant ($P < 0.01$).

Aside from the inter-gene correlation analyses, analyses between $\log_{10}$-transformed water quality data and relative gene abundances were also performed, with no strong correlations observed (data not shown). However, of these correlations, moderately strong positive relationships ($0.6 < r < 0.7$) were observed between electrical conductivity and *intI1* and *sul1* ($P < 0.001$). Surprisingly, no strong correlations were observed between dissolved nitrate and relative gene abundances, as we noted previously that average relative ARG and *intI1* abundances were statistically greater in IRFs that received drain tunnel outflow with increased nitrate loading. In addition to the chemical and physical parameters presented in Table 1, we also attempted to quantify antibiotic residues in each set of grab water samples. We did have some limited detections for chlortetracycline, oxytetracycline, tetracycline, lincomycin, sulfadimethoxine, sulfamethazine, sulfamethoxazole, sulfathiazole, and trimethoprim, but in most cases the levels were at or slightly above the detection limit of 1.0 ng L^{-1} (data not shown). Consequently we were unable to perform correlation analyses between the genes and antibiotic residue levels. A quantitative effect of environmentally relevant antibiotic concentrations on the development of antibiotic resistance in water bacteria has not been elucidated to date and is complicated by the fact that ARGs are found in environments that are essentially free of anthropogenic antibiotics (Aminov, 2009; Baquero et al., 2008; Zhang et al., 2009).

4. Conclusions

The presence of elevated levels of ARGs and their dissemination in the environment, including those targeted in this study, as well as numerous other ARGs that have been described in the literature, are a potential risk to human health if they are acquired by pathogens. As a result, it is useful to monitor ARGs to determine if particular anthropogenic activities are influencing their occurrence and abundance in environmental settings. Certain environmental hotspots are suspected of contributing to the global spread of antibiotic resistance, thus improving our overall understanding of these potential problem areas can help determine if targeted mitigation strategies are necessary. In the present study, ARGs of clinical relevance were detected in return flows at levels that generally exceeded background levels found in canal water that is used for irrigation in the USR watershed. The IRF sites with the greatest relative abundances were those that received subsurface drainage from tunnels used to prevent waterlogging of irrigated fields. These results suggest that irrigated croplands with drain tunnels are a major source of ARGs in the IRFs, although the contribution from non-agricultural sources should not be ruled out. The most likely scenario is that ARGs and antibiotic resistant bacteria entrained in manure and biosolids, that are land applied as fertilizers, are mobilized during irrigation/rainfall events and make their way to the IRFs in surface runoff and subsurface drainage. However, without source tracking methods for resistance genes it is not possible to know if they were from human and/or animal sources. Regardless of the ARG source, the fact that most of the IRFs contained higher levels than found in the canal water, indicates that IRFs can be a point source of ARGs that ultimately discharge into the Snake River. Whilst it is not known if the ARGs present an increased risk to human health, this dataset will be useful to risk assessors and those interested in understanding the environmental dimensions of antibiotic resistance in watersheds. It was also found that ARG levels were not strongly correlated (although significant in many instances) with the class 1 integrase-integron gene, nor IncP-1 and IncQ-1 plasmids, suggesting that the ARGs were not enriched as a result of horizontal gene transfer among or replication within environmental bacteria. This study increases knowledge that IRFs accumulate ARGs from the surrounding land base and moves them from the watershed to receiving waterbodies, such as rivers, further enhancing their dissemination in the environment.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2019.113568.

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