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Effect of River Landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG)

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ABSTRACT

The purpose of this study was to quantify antibiotic resistance genes (ARG) in the sediments of the mixed-landscape Cache La Poudre River, which has previously been studied and shown to have high concentrations of antibiotics related to urban and agricultural activities. River sediments were sampled during two events (high-flow and low-flow) from five sites with varying urban and agricultural impact levels. Polymerase-chain-reaction (PCR) detection assays were conducted for four sulfonamide resistance gene families, using newly designed primers, and five tetracycline resistance gene families, using previously published primers. *Sul*(I), *sul*(II), *tet*(W), and *tet*(O) gene families were further quantified by real-time quantitative polymerase chain reaction (Q-PCR). Resistance to four classes of antibiotics (tetracyclines, sulfonamides, ionophores, and macrolides) was also investigated using a culture-based approach. The quantities of resistance genes normalized to the 16S gene copy number were significantly different between the sites, with higher resistance gene concentrations at the impacted sites than at the pristine site. Total resistant CFUs were over an order of magnitude lower at the pristine site, but differences were less apparent when normalized to the total CFUs. Six tetracyclines and six sulfonamides were also quantified in the sediments and were found to be highest at sites impacted by urban and agricultural activity, with no antibiotics detected at the pristine sit. To the knowledge of the authors, this study is the first to demonstrate a relationship between urban and agricultural activity and microbial resistance in river sediments using quantitative molecular tools.

1. Introduction

Pharmaceutical compounds including antibiotics, hormones, and steroids are widely used to prevent and/or treat diseases and to promote animal growth in livestock production facilities, such as concentrated animal feeding operations (CAFOs). In particular, one half of the 50 million pounds of antibiotics produced each year in the US is used for agriculture and 90% of these are used for growth promotion (Levy, 1998). A significant amount of these antibiotics (up to 75%; Elmund et al., 1971; Feinman and Matheson, 1978) are excreted as active metabolites. Thus, animal waste presents a major potential source of antibiotic input to the environment (Haapapuro et al., 1997; Sweeten, 1992). Discharge of treated municipal, hospital, and veterinary wastewaters present additional sources. A recent study conducted by the United States Geological Survey in 1999 and 2000 indicated that, out of a network of 139 streams across 30 states, 95 contained

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antibiotics (Kolpin et al., 2002). Now that antibiotic contamination in the environment has been confirmed, a growing concern is that the release of antibiotics into the environment may contribute to the emergence of strains of disease-causing bacteria that are resistant to even high doses of these drugs (ASM, 2002; Chee-Sanford et al., 2001; Goni-Urriza et al., 2000; Guardabassi et al., 1998). Therefore, there is a need to better understand the relationship between antibiotics in the environment and their ultimate impact on the emergence of microbial resistance.

Recently, the occurrence of four classes of antibiotics (tetracyclines, sulfonamides, macrolides and ionophores) has been investigated in environmental samples such as natural water, animal and human wastewaters, irrigation ditches, and river sediments (Kim and Carlson, 2005; Yang and Carlson, 2003, 2004a,b; Yang et al., 2004). In particular, within the mixed-landscape of the Cache La Poudre (Poudre) River watershed, the presence of these four classes of antibiotics in water and sediments were found to relate to urban and agricultural activity. Generally, the number of antibiotics present and their concentrations increased as agricultural and urban activates increased along the Poudre River from its pristine origins in the Rocky Mountains, where no antibiotic contamination has yet been found. Relationships could also be identified between antibiotics known to be specific to human or agricultural use, and their most likely inputs (Yang and Carlson, 2003, 2004a,b; Yang et al., 2004). Based on these results it is clear that studies are needed to assess the impact of antibiotics released into the environment and their potential ultimate effect on human health and the environment. In particular, there is a need for the development and application of methods to quantify actual resistance genes in the environment. The quantity of antibiotic resistance genes (ARG) may serve as a good indicator of the bioavailability of antibiotics in the environment. Also, as suggested recently by Rysz and Alvarez (Rysz and Alvarez, 2004), the genes themselves could be considered to be "pollutants," as their wide-spread dissemination is clearly undesirable.

The purpose of this study was two-fold: (1) to develop methods for quantifying ARG in environmental samples; and (2) to apply these methods in assessing and quantifying ARG present in the sediments of the Poudre River, a model river system that is zonated with respect to pristine, urban, and agricultural impacts. In particular, quantitative real-time polymerase chain reaction (Q-PCR) methods targeting tetracycline and sulfonamide resistance genes were developed and applied. Both of these classes of antibiotics are widely used in both animals and in humans (Aminov et al., 2001; Huber, 1971; Pereten and Boerlin, 2000). A culture-based approach was also implemented as a broad method of quantifying microbial resistance, including resistance incurred by genes that may not have been previously described. Finally, various tetracyclines and sulfonamides were also quantified in the sediment samples in order to compare the concentrations of antibiotics observed with those of ARG.

2. Materials and methods

2.1. Study area and sampling sites

The Poudre River in northern Colorado has many unique attributes that make it an excellent model watershed to compare pristine, urban, and agricultural impacts. These attributes include: (1) the semi-arid nature of the front range of Colorado which results in only a small number of tributaries to the Poudre River; (2) the predominance of point sources in the urban landscape of Fort Collins versus non-point sources in the agricultural areas outside of the city; and (3) the source of the river being primarily snowmelt with minimal anthropogenic influences (Yang and Carlson, 2003, 2004b).

Five sites along the Poudre River were monitored and are summarized in Table 1. A map of this study site has been previously published (Yang and Carlson, 2003). Sample site 1 was chosen to represent the pristine area without anthropogenic influence and is located at the river origin. The river originates near the continental divide in the Rocky Mountain National Park and flows through steep mountainous terrain for approximately 43 miles before entering the front range city of Fort Collins. To date, no antibiotic compounds have been detected at site 1 (Kim and Carlson, 2005; Yang and Carlson, 2003, 2004a,b; Yang et al., 2004). Site 2 is located just prior to where the river enters Fort Collins and represents an area influenced by light agricultural activities (a few small-scale dairies and cattle feed operations). Sample site 3 is located at the point of discharge for the Drake Wastewater Reclamation Facility after the river travels through the city and represents an urban area influenced by wastewater discharge. Sample site 4 is downstream of Fort Collins but upstream of the next urban area, Greeley, CO. Between Fort Collins and Greeley there are at least six CAFOs and multiple smaller animal feed facilities, therefore, site 4 represents an area heavily influenced by agriculture activities. Site 5 is downstream of Greeley and represents both urban and

Table 1 – Poudre River sampling site locations and characteristics

Site ID	Actual location	Site characteristics
Site 1	Greyrock National Recreation Trail, Ft Collins, CO	Pristine area without anthropogenic influence
Site 2	Shields street bridge, Ft Collins, CO	Area influenced by light agriculture activities
Site 3	Drake waste water treatment plant, Ft Collins, CO	Urban area influenced by wastewater discharges
Site 4	Hwy 329 bridge, Windsor, CO	Area influenced significantly by agriculture activities
Site 5	Weld country municipal airport, Greeley, CO	Area influenced by both urban and agriculture activities

agriculture influences since Greeley is home to several CAFOs near the town and a beef processing plant that processes up to 3000 animals per day. Previous work has documented the occurrence of several different classes of antibiotic compounds, including: tetracyclines; sulfonamides; macrolides; β-lactams; and ionophores, some are as high as the microgram per kilogram range in the most heavily impacted sites 3–5 (Kim and Carlson, 2005; Yang and Carlson, 2003, 2004a,b; Yang et al., 2004).

2.2. Sampling

Sediment samples were collected in April 2004 (high-flow, 6.8 cm) and February 2005 (low-flow, 0.8 cm) along the Poudre River at the five sites. The upper sediments (about 5 cm) from the middle and two sides of a cross-section at each site were sampled and composited. Samples were collected using a shovel and mixed well in sterilized centrifuge tubes. Fifty-five grams of mixed sample at each site were stored at −80 °C for subsequent molecular analysis. The remainder of the samples were processed within 8 h for viable culturing.

2.3. Heterotrophic plate counts on antibiotic-selective media

10× fold serial dilutions were prepared with a 10^{-1} dilution defined as 1 g of sediment sample diluted in 9 mL of sterilized phosfate-buffered saline (PBS, pH = 7.4). Diluted sediment samples were directly plated onto R2A agar medium (Difco, Sparks, MD) dosed with and without antibiotics. The concentrations of antibiotics were: chlortetracycline (CTC) 70.55 mg/L; oxytetracycline (OTC) 45.55 mg/L; meclocycline (MCC) 560.4 mg/L; sulfamethazine (SMT) 281.8 mg/L; sulfamethoxazole (SMX) 50.4 mg/L; erythromycin (Ery) 18.1 mg/L; tylosin (Tyl) 547.45 mg/L and monensin (Mon) 11 mg/L. For water soluble antibiotics, the concentrations were chosen as five times the average reported LD_{50} values, whereas for insoluble antibiotics (SMX, SMT, Mon, MCC and OTC) the concentrations were chosen as the maximum amount that dissolved readily in water and when subsequently added to the melted agar did not affect solidification. Plates were incubated at 37 °C for 48 h, and thereafter at room temperature (shielded from light to prevent antibiotic degradation) for another week before determining the colony forming units (CFUs). This was in order to ensure that slow-glowing organisms were included.

2.4. DNA extraction and purification

DNA was extracted from sediment samples using the FastDNA Spin Kit for Soil (QBiogene, Carlsbad, CA). To aid in downstream quantification, exactly 0.5 g of sample from each site was used for DNA extraction. The steps were followed according to the protocol provided by the manufacturer. DNA was further purified using the Geneclean Spin Kit (QBiogene, Carlsbad, CA) to minimize PCR inhibition. The concentration of DNA before and after purification was determined and the percent recovery was recorded.

2.5. Primer design

All currently available nucleotide sequences encoding sulfonamide resistant genes were downloaded from the GenBank Database (http://www.ncbi.nlm.nih.gov/). Proteins, antibiotic resistance islands, and plasmids encoding sulfonamide resistance genes were included mainly from pathogenic carriers, including: *Typhimurium* sp., *Salmonella typhimurium, Salmonella enterica, Streptomyces coelicolor, Streptococcus pneumoniae, Corynebacterium diphtheriae, Escherichia coli, Photorhabdus luminescens, Enterica serovar, Actinobacillus pleuropneumoniae,* and *Pasteurella multocida*.

Sequences were aligned with the multiple-sequence alignment program CLUSTALX 1.81 (Thompson et al., 1997). Sequences within clusters were separately aligned and compared with each other in order to create consensus sequences for the primer design templates using FastPCR (Kalendar, 2004). The size of the PCR product was specified in the range of 100–350 bp for Q-PCR suitability. Specificity was verified using the BLAST alignment tool (http://www.ncbi.nlm.nih.gov/blast/). Purified PCR products obtained from sediment DNA extract were cloned and sequenced in order to further confirm specificity. The four sets of *sul* primers from which verifiable target products were obtained are shown in Table 2.

2.6. PCR assays for detection of resistance genes

Qualitative PCR assays were performed in order to determine which of the four sulfonamide ARG and the five tetracycline ARG encoding ribosomal protection (Aminov et al., 2001) were detectable at the sites. These assays were carried out using the Eppendorf MasterTaq kit (Eppendorf, Westbury, NY) in a 25 μL volume reaction. The PCR master mixture consisted of 5 μL 5× buffer; 2.5 μL 10× buffer; 0.2 mM dNTPs; 0.2 μM primers; 1.75 units of Taq DNA polymerase, and 1 μL of template. The temperature program consisted of initial denaturing at 95 °C, followed by 40 cycles of 15 s at 95 °C; 30 s at the annealing temperature (Table 2 for *sul* genes, 60 °C for *tet*(W), 50.3 °C for *tet*(O), 56 °C for *tet*(S), 43.9 °C for *tetB*(P), 43.9 °C for *tet*(T)—note these vary from Aminov et al. (2001)); 30 s at 72 °C, and a final extension step for 7 min at 72 °C.

2.7. Real-time quantitative PCR (Q-PCR)

Q-PCR protocols were developed using the intercalating dye SYBR Green I (Molecular Probes, Inc., Eugene, OR) to transition from traditional PCR to real-time Q-PCR. Because SYBR Green I binds non-specifically to all double-stranded DNA, appropriate measures were taken to ensure that the signals obtained were from specific products rather than primer–dimer or other non-specific products. Q-PCR protocols were optimized to quantify sulfonamide resistance genes *sul*(I) and *sul*(II), using the designed primers, and *tet*(W) and *tet*(O), using previously described primers (Aminov et al., 2001; Chee-Sanford et al., 2001). Q-PCR reactions were performed with a SmartCycler (Cepheid, Sunnyvale, CA) in a 25 μL reaction mixture (1× SYBR Green PCR Master Mix (Qiagen, Valencia, CA), 0.2 μM of each primer, and 1 μL of template) with a temperature program of 15 min at 95 °C (initial denaturing and Hot Start Taq activation), followed by 50 cycles of 15 s at

Table 2 – PCR primers targeting sulfonamide resistance gene families

Primer	Class targeted	Sequences	Traditional PCR annealing temp (°C)	Q-PCR annealing temp (°C)	Amplicon size (bp)
*sul*I-FW[a]	*sul*(I)	cgcaccggaaacatcgctgcac	55.9	65.0	163
*sul*I-RV		tgaagttccgccgcaaggctcg			
*sul*II-FW	*sul*(II)	tccggtggaggccggtatctgg	60.8	57.5	191
*sul*II-RV		cgggaatgccatctgccttgag			
*sul*III-FW	*sul*(III)	tccgttcagcgaattggtgcag	60.0	61.0	128
*sul*III-RV		ttcgttcacgccttacaccagc			
*sul*A-FW	*sul*(A)	tcttgagcaagcactccagcag	60.0	60.0	299
*sul*A-Rv		tccagccttagcaaccacatgg			

[a] FW, forward; RV, reverse.

95 °C; 30 s at the annealing temperature (65 °C for *sul*(I), 57.7 °C for *sul*(II), 60 °C for *tet*(W), and 50.3 °C for *tet*(O)); and 30 s at 72 °C (optical window on) followed by a final melt curve stage with temperature ramping from 60 to 95 °C.

2.8. Q-PCR standard curves and quantification

To generate positive controls for calibration, genes from the site sediment DNA extractions were PCR-amplified using traditional PCR and cloned using the TOPA TA Cloning Kit (Invitrogen, Carlsbad, CA). The log copy number of genes per μL DNA template solution for y-axis of the calibration curve was calculated by

$$\text{Log copy of genes}/\mu\text{L DNA} = \log \frac{b \times c}{L \times a \times 10^{12}}, \quad (1)$$

In which, a is the weight of kb DNA per pmol (1 kb DNA = 0.66 μg/pmol), b the Avogadro's constant (6.022×10^{23}/mol), L the length of template containing the target gene, c the concentration of template in μg/μL.

Two approaches were taken to investigate the effect of the DNA extraction matrix and the potential for threshold cycle (C_T) value suppression. (1) To determine if the concentration of a background DNA affects the amplification of the desired genes, two concentrations (140 ng/uL and 50 ng/μL) of additional non-target calf thymus DNA (BioRad, Hercules, CA) was spiked into the calibration control templates. Ten-fold serial dilutions of both solutions (140 ng/μL *Calf thymus*+3 ng/μL desired gene and 50 ng/μL *Calf thymus*+3 ng/μL desired gene) were made up to 10^{-10} to determine the detection limit. For each type of gene, at least six replicates of 10 different concentrations at two carrier DNA levels were used to create the standard curves. The detection limit for each gene was determined by the maximum dilution that produced a consistent C_T value (within 5%). (2) The impact of PCR inhibitors potentially present in the DNA extract from the sediments was also investigated. DNA extract from each of the five sites was spiked with known amounts of template and the difference in C_T value between the matrix and the control was determined. The matrix test was performed for each of the four resistance genes at two different concentrations of spiked template (10× and 100× of the concentration of the sediment DNA) and a consistent value was obtained for each DNA with respect to the percent suppression observed.

Correlation coefficients were more than 99% ($R^2 > 0.99$) for all calibration curves, indicating that the relationship between the C_T value and the log copy number of genes per Q-PCR reaction was linear over 8 orders of magnitude. The levels of detection (LOD) for *sul*(I), *sul*(II), *tet*(W), and *tet*(O) were observed to be 8, 7, 1, and 1 copies of genes per 25 μL assay, respectively, which were then implemented as the level of quantification (LOQ) in the calibration curves. It was determined that the background DNA concentration had no significant effect on the amplification of target genes, as all C_T values fell within 5% of each other, regardless of the concentration of calf thymus DNA. However, the matrix study revealed that suppression of the C_T value by the DNA extract matrix was apparent and varied between sites. Little to no suppression was observed in DNA extract from the pristine site, while the matrix from the impacted sites lowered the gene copy number estimate by as much as 20× and an average of 4×. A suppression factor was determined for each DNA matrix and primer combination as noted in Eq. (2), where c is the number of templates enumerated in the control, t is the number of templates enumerated in DNA extraction matrix, and m is the number of templates enumerated when the same control is spiked into the DNA extraction matrix:

$$S = (c + t) \div m. \quad (2)$$

Quantification of ARG at the sites thus took into account the mass of sediment extracted (0.5 g), any losses observed during DNA cleaning steps (as a fraction), and the PCR suppression factor. Therefore, the copy of genes/gram of sediment was determined according to

$$\frac{\text{gene copies}}{\text{g sediment}} = d \times \left(\frac{50\,\mu\text{L DNA extract}}{0.5\,\text{g sediment}}\right) \times e \times S, \quad (3)$$

where d is the copy of genes/μL DNA extract determined from calibration curve, e is the correction factor for losses during cleaning the DNA extract (1/loss fraction), and S is the matrix suppression factor (Eq. (2)). Although several kits and conditions were tested for Q-PCR, in general, it was found that Q-PCR was more susceptible to DNA extraction matrix

inhibition and thus the remaining genes were detected by traditional PCR, but could not be quantified by Q-PCR.

In order to account for potential changes in the overall size of the microbial population at the sites, a standard curve was generated using "universal" primers and a TaqMan probe targeting Bacterial 16S rDNA as described in Suzuki et al. (2000).

2.9. Quantification of antibiotics

For quantification of antibiotics in sediments, McIlvaine Buffer solution (pH 4.0) was used to pre-extract tetracyclines and sulfonamides from the solid phase to the liquid phase, followed by solid phase extraction (SPE) to clean-up and concentrate the sample. High-performance liquid chromatography tandem mass spectrometry (HPLC/MS/MS) operated in positive mode with electrospray ionization (ESI) was used to separate and detect the tetracyclines and sulfonamides. Detailed information on the quantification of tetracyclines and sulfonamides in sediments is described elsewhere (Kim and Carlson, 2005).

3. Results

3.1. Heterotrophic plate counts on antibiotic-selective media

In terms of absolute numbers of resistant CFU per gram of sediment summed for the eight antibiotic R2A media, the pristine site was found to be the lowest at 1.9×10^3 CFU/gram sediment for the high-flow sampling event and 5.7×10^4 CFU/gram sediment for the low-flow event. Sites 2 and 4 were the next lowest, with site 2 at 2.0×10^5 and 4.4×10^6 CFU/gram sediment for the high-flow and low-flow events, respectively, and site 4 at 1.0×10^5 and 1.6×10^7 CFU/gram sediment, respectively. Sites 3 and 5 were the highest for both the high-flow and low-flow events, at 1.1×10^6 and 2.4×10^7 CFU/gram sediment, respectively, for site 3, and 2.5×10^6 and 5.6×10^7 CFU/gram sediment, respectively, for site 5. For all sites the number of resistant CFUs was higher during the low-flow sampling event.

Because the total CFU growing on control plates varied significantly among the sites (3 orders of magnitude difference between sites 1 and 5 for both events), there was no strong contrast between the five sites when the resistant CFU data were normalized to the control CFU. The normalized heterotrophic CFU data are plotted in Fig. 1 for the high-flow sampling event and Fig. 2 for the low-flow sampling event. A high level of resistance to monensin, an ionophore antimicrobial exclusively used in agriculture, was noted at sites 3–5, especially during the high-flow event.

3.2. PCR assays for detection of resistance genes

The qualitative occurrence data for tetracycline and sulfonamide resistance gene families at the sites obtained by PCR assays are summarized in Table 3. During the high-flow event it was readily apparent that the number of different sulfonamide and tetracycline ARG detected increased as activity at the sites increased. Statistical analysis of the binomial data fit to a generalized linear model indicated that sites 1 and 5 ($p = 0.01$) and sites 2 and 5 ($p = 0.01$) were

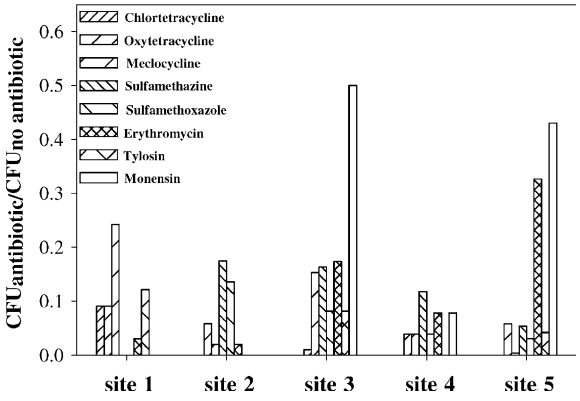

Fig. 1 – CFUs on antibiotic media normalized to CFUs on media with no antibiotic added for the high-flow sampling event.

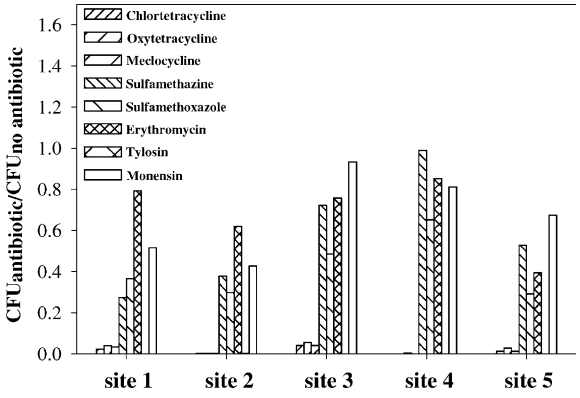

Fig. 2 – CFUs on antibiotic media normalized to CFUs on media with no antibiotic added for the low-flow sampling event.

significantly different for the high-flow sampling event, and that there was no statistical difference between the most heavily impacted sites. In contrast, there was no significant difference between any of the sites during the low-flow event, and only *sul*(II) was detected exclusively at the three most heavily impacted sites.

3.3. Quantification of resistance genes at the sites

The numbers of copies of the four resistance genes quantified at each site are plotted normalized to the number of copies of Bacterial 16S rRNA genes in Fig. 3 for the high-flow and Fig. 4 for the low-flow sampling events. Normalizing to the number of copies of 16S rRNA genes provided a means to assess the level of resistance proportional to the size of the overall population. The total number of copies of Bacterial 16S rRNA genes was found to be relatively consistent between sites (within 1 order of magnitude, except site 2, which was 1–2 orders of magnitude lower for both events).

The total concentrations of *sul*(I) and *sul*(II) together were the highest at sites 3 and 5, where there was heavy influence from both urban and agricultural activity. *Sul*(I) and *sul*(II) were present at site 4, but significantly lower in concentration

Table 3 – Detection of resistance gene families at sampling sites

Gene ID	April, 2004 high-flow sampling event					February, 2005 low-flow sampling event					+ Control
	Site 1	Site 2	Site 3	Site 4	Site 5	Site 1	Site 2	Site 3	Site 4	Site 5	
tetB(P)	–	–	–	–	+	–	–	–	–	–	+
tet(O)	+	–	+	+	+	+	+	+	+	+	+
tet(S)	–	–	–	–	+	–	–	–	–	–	+
tet(T)	–	–	+	+	+	–	–	–	–	–	+
tet(W)	–	+	+	+	+	+	+	+	+	+	+
sul(I)	+	+	+	+	+	+	+	+	+	+	+
sul(II)	–	–	+	+	+	–	–	+	+	+	+
sul(III)	–	–	+	+	+	–	–	–	–	–	+
sul(A)	–	–	+	–	+	–	–	–	–	–	+

+: present; –: absent.

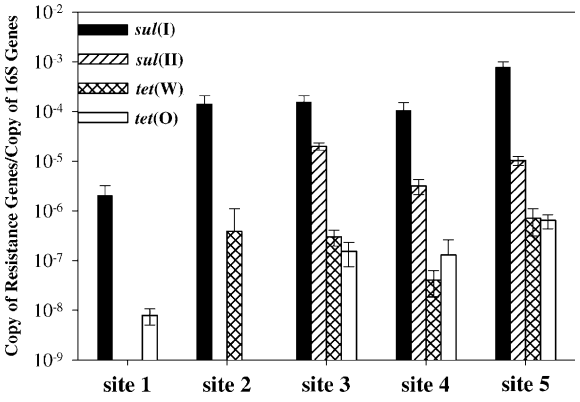

Fig. 3 – Copies of resistance genes normalized to the number of Bacterial 16S rRNA genes at different sites for the high-flow sampling event. Error bars indicate the standard deviation of six replicates in three independent Q-PCR runs.

than they were at sites 3 and 5. Site 2 showed concentrations of *sul(I)* equal to or greater than those observed at sites 3 and 5, however, *sul(II)* was consistently absent. *Sul*(II) was also absent from site 1 in both sample events, while *sul*(I) was 2–3 orders of magnitude lower during the high-flow event, and below Q-PCR detection in the low-flow event.

In the high-flow sampling event, the total *tet* genes followed the same trend as the total *sul* genes, with sites 3 and 5 the highest, followed by site 4, and detection of only one of the pair of genes at sites 2 and 1. In the low-flow event, however, there was no significant difference between site 1 and any of the other sites for *tet*(O), and *tet*(W) was significantly different (lower) only at site 1. *Tet*(W) and *tet*(O) genes were lower in concentration than *sul* genes at all sites.

3.4. *Quantification of antibiotics*

The concentrations of tetracycline and sulfonamide antibiotics found in the sediments are presented in Table 4. As has been observed in previous studies, the concentrations of tetracyclines and sulfonamides were highest at sites 3–5. It was also observed that the antibiotic concentrations were higher during the low-flow sampling event. Correlation analyses conducted comparing the concentration of the antibiotics in the sediments and concentrations of genes did not reveal statistically significant relationships for either the high-flow or the low-flow sampling events (R^2 ranged from 0.12 to 0.84, *p*-value ranged 0.92–0.47).

4. Discussion

4.1. *Applicability of the methods*

The Q-PCR methods developed in this study provide a useful means to quantify ARG in the environment and thus bioassay and monitor the relative impact of antibiotic use. Q-PCR also allows for direct quantification of ARG as a new class of "pollutants" in and of themselves. While all three levels of data gathered in this study (CFU, PCR presence/absence assay, Q-PCR) revealed similar overall trends, there were some differences observed. For example, the CFUs on control plates without antibiotics varied over 3 orders of magnitude between sites, while the quantities of 16S rRNA genes were relatively consistent between sites. Because PCR of 16S rRNA genes would quantify the DNA of both live and dead cells, it cannot be determined whether the culturing approach was biased, or if there were varying proportions of 16S rRNA genes corresponding to dead cells at the five sites. In particular, this affected interpretation of the data at site 1, where the CFU growing on control plates were lowest and thus provided a high estimate of the proportion of resistant CFU. However, site 1 is also the most pristine ecologically and therefore would be expected to be the least amenable to growth on a Petri dish. On the other hand, 16S rRNA genes are well-studied and increasingly being used as biomarkers for normalizing quantitative data in the environment. The Q-PCR method also is less cumbersome and provides results within 3 h, including DNA extraction.

In applying Q-PCR to environmental samples, however, one needs to be aware of the potential impacts of PCR inhibitors.

Table 4 – Concentrations of tetracyclines and sulfonamides at sampling sites

	April, 2004 high-flow sampling event (μg/L)					February, 2005 low-flow sampling event (μg/L)				
	Site 1	Site 2	Site 3	Site 4	Site 5	Site 1	Site 2	Site 3	Site 4	Site 5
Tetracycline (TC)	ND	3.6	8.7	8.4	10.2	ND	11.0	102.7	3.9	24.9
Chlortetracycline (CTC)	ND	3.0	3.1	4.6	3.8	ND	9.6	19.3	15.8	22.0
Oxytetracycline (OTC)	ND	2.4	7.3	7.4	23.6	ND	7.8	56.1	19.0	35.5
Demeclocyline (DMC)	ND	2.1	6.8	2.1	6.9	ND	6.5	14.7	9.5	23.5
Meclocycline (MCC)	ND	29.5	21.6	28.4	41.6	ND	38.3	167.5	26.3	72.0
Doxycycline (DXC)	ND	5.1	10.2	6.3	14.8	ND	13.3	38.9	12.0	25.6
Sum of tetracyclines	ND	45.7	57.7	57.2	100.9	ND	86.5	399.1	86.4	203.5
Sulfathiazole (STZ)	ND	2.7	4.4	3.5	4.7	ND	2.7	4.4	3.5	4.7
Sulfamerazine (SMR)	ND	ND	ND	ND	ND	ND	1.7	15.0	ND	ND
Sulfamethazine (SMT)	ND	1.7	1	ND	ND	ND	ND	ND	ND	ND
Sulfachloropyridazine (SCP)	ND	ND	3	ND	ND	ND	ND	3.0	ND	ND
Sulfamethoxazloe (SMX)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Sulfadimethoxine (SDM)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Sum of sulfonamides	ND	4.4	8.4	3.5	4.7	ND	4.4	22.4	3.5	4.7

ND: Not detected.

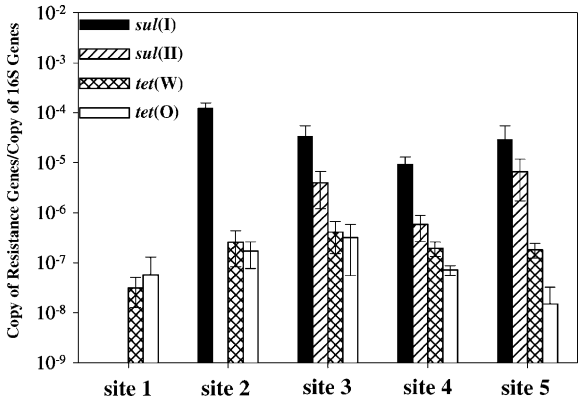

Fig. 4 – Copies of resistance genes normalized to the number of Bacterial 16S rRNA genes at different sites for the low-flow sampling event. Error bars indicate the standard deviation of six replicates in three independent Q-PCR runs.

In this study there were some genes detected by the PCR assay that could not be quantified by Q-PCR because Q-PCR was apparently more sensitive to inhibitors. Inhibitors present in the DNA extraction matrix were also a factor in Q-PCR in that they lowered the estimated gene concentration by an average of 4 × and as high as 20 × in one case. However, this was easily corrected for by determining the suppression factors in spiked matrix control tests.

4.2. *Land-use and occurrence of resistance*

To the knowledge of the authors, this study is the first report of an increase in ARG in river sediments corresponding to increases in human and agricultural activity. This is consistent with the findings of previous studies that have shown that as the Poudre River runs through pristine, urban, and agriculturally influenced areas, the concentrations of various antibiotic compounds increase (Kim and Carlson, 2005; Yang and Carlson, 2003). To date, no antibiotics have been detected at site 1 which is considered not to have any anthropogenic influences, and the highest concentrations of antibiotics have been found at sites 3–5, which are the most heavily impacted. Similarly, the kinds and quantities of resistance genes detected at site 1 were consistently lower at site 1, in most cases by orders of magnitude. It should be noted that detection of resistance genes at site 1 was not unexpected considering that resistance genes are naturally occurring. This was also consistent with the observed growth of some heterotrophic bacteria on plates with sulfonamides and tetracyclines. The characteristics of site 1 thus provided a good means of characterizing the background level of resistance for comparison to impacted sites.

Other researchers have recently investigated the presence of resistance genes in animal feedlot lagoons and areas immediately adjacent. For example, Chee-Sanford et al. (2001) demonstrated a high occurrence of tetracycline resistance genes in the groundwater underlying swine production facilities using traditional PCR assays. More recently, Smith et al. (2004) developed a TaqMan Q-PCR assay for *tet*(W), *tet*(O), and *tet*(Q) and observed a correlation with the concentrations of these genes and the concentration of tetracyclines in 18 different lagoons. The present study further supports such findings and takes the ARG dissemination pathway a step further by documenting their occurrence in impacted river sediments.

The lack of a statistically significant correlation in this study between the concentration of antibiotics in the sediments and the concentration of resistance genes could be the result of various factors. For example, other types of resistance genes may have been present, but not analyzed. In this case, while numerous tetracycline and sulfonamide ARG have been reported in the literature, PCR primers were

developed or applied for only 15 *sul* and *tet* genes total, nine of which tested positive in the PCR assay, and only four were quantified. To fully characterize the correlation, full discovery and quantification of all resistance genes would be required. It should also be pointed out that microbial populations may retain resistance genes long after their initial exposure, which could additionally contribute to a lack of direct correlation. Also, lower concentrations of antibiotics may actually enhance the development of resistance, considering that very high concentrations are often lethal even to resistant cells. Such phenomena would be of significant interest in developing a model of understanding of the spread of ARG and strategies for minimizing their impact. Recent work investigating the fate of antibiotic resistant organisms in column studies suggests that about one month was required for resistance levels to return to baseline after tetracycline was removed from the feed (Rysz and Alvarez, 2004).

4.3. *Sulfonamide resistance genes*

Sulfonamides are used both in animals and in humans, though generally more-so in humans. This is because sulfonamides are used primarily as therapeutic agents, rather than as routine growth-promoters. The target of sulfonamide antibiotics is the enzyme dihydropteroate synthase (DHPS) in the folic acid pathway. *Sul*(I) and *sul*(II) are two alternative sulfonamide resistant DHPS genes found in Gram-negative bacteria (Sköld, 2000). In a case study of pathogenic *E. coli* from various livestock in Switzerland by Lanz et al. (2003), about 70% of the sulfonamide-resistant isolates from pigs could be explained by the presence of *sul*(I) and *sul*(II). Both of these genes have also been detected in human pathogens such as *Salmonella typhimurium*, *E. coli*, and *Streptococcus pneumoniae*. The most commonly detected sulfonamide resistance genes in this study were also mainly *sul*(I) and *sul*(II), and based on the research of Lanz et al., their likely inputs may be from the CAFOs that are prolific in northern Colorado.

4.4. *Tetracycline resistance genes*

Tet(W) and *tet*(O) are both common in anaerobic intestinal and rumen environments and both incur resistance by ribosomal protection mechanisms (Barbosa et al., 1999; Taylor et al., 1987). *Tet*(O) is found in both Gram-positive and Gram-negative bacteria (Roberts, 1997), and approximate estimates suggest that up to 5% of the bacteria in bovine rumen and swine intestines may carry the *tet*(O) gene (Aminov et al., 2001). *Tet*(W) is also found in bovine and sheep rumen as well as human intestinal isolates (Melville et al., 2004; Scott et al., 2000). In a study by Smith et al. (25) *tet*(O) and *tet*(W) along with *tet*(Q) were the most commonly found tetracycline resistance genes in 18 different feedlot lagoons. Thus, the presence of the *tet*(W) and *tet*(O) resistance genes may be a good indicator of fecal contamination from humans and/or animals. If *tet*(O) and *tet*(W) are truly indicators of fecal contamination, then the present study suggests that such contamination is wide-spread at the impacted sites, especially during high-flow events, which may be the result of transport of fecal bacteria in runoff. *Tet* genes may have been lower at site 5 during the low-flow event, even though the concentration of tetracyclines was double, because there was less runoff. On the other hand, both *tet*(O) and *tet*(W) have been cited as being promiscuous in their ability to spread among and across populations (Billington et al., 2002; Smith et al., 2004) and *tet*(W) in particular has recently been identified as being associated with a conjugative transposon (Melville et al., 2004). Examples of *tet*(W) carrying strains include *Mitsuokella multiacidus*, *Selenomonas ruminantium*, and *Butyrivibrio fibrisolvens* (Barbosa et al., 1999). Further investigation is needed in order to determine whether horizontal gene transfer interferes with the use of these genes as indicators of fecal sources of ARG.

5. Conclusions

This study demonstrates a relationship between human and agricultural activity and elevated levels of ARG in river sediments using quantitative molecular tools. This is an important step in developing a paradigm for ARG as environmental contaminants. Further study is needed in order to better understand the relationship between the use of antibiotics in humans and animals and the spread of ARG. In particular, the relationship between the transport of antibiotics and the transport of ARG in the environment has not been defined. For example, in this study it is not clear whether the elevated levels of resistance quantified in the river sediments were a result of antibiotic selection taking place in the sediments, or if selection occurred upstream and the resistant microbes were later transported there.

Another important area of future work will be to build a better understanding and separation of human and animal sources. Source tracking methods for resistance genes need to be further developed and applied to accomplish this. Even if it becomes possible to clearly distinguish human and agricultural sources of ARG, it will still be important to consider that even antibiotics used exclusively for agriculture have been demonstrated to co-select for microbial resistance to a variety of antibiotics. A better understanding of the sources and fates of antibiotics and ARG will facilitate improved modeling of the ultimate impact of antibiotic use on human, animal, and environmental health and may ultimately be applied in developing strategies to mitigate potentially adverse impacts.

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