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Antibiotic Resistance Genes as Emerging Contaminants: Studies in Northern Colorado†

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This study explores antibiotic resistance genes (ARGs) as emerging environmental contaminants. The purpose of this study was to investigate the occurrence of ARGs in various environmental compartments in northern Colorado, including Cache La Poudre (Poudre) River sediments, irrigation ditches, dairy lagoons, and the effluents of wastewater recycling and drinking water treatment plants. Additionally, ARG concentrations in the Poudre River sediments were analyzed at three time points at five sites with varying levels of urban/agricultural impact and compared with two previously published time points. It was expected that ARG concentrations would be significantly higher in environments directly impacted by urban/agricultural activity than in pristine and lesser-impacted environments. Polymerase chain reaction (PCR) detection assays were applied to detect the presence/absence of several tetracycline and sulfonamide ARGs. Quantitative real-time PCR was used to further quantify two tetracycline ARGs (tet(W) and tet(O)) and two sulfonamide ARGs (sul(I) and sul(II)). The following trend was observed with respect to ARG concentrations (normalized to eubacterial 16S rRNA genes): dairy lagoon water > irrigation ditch water > urban/agriculturally impacted river sediments ($p < 0.0001$), except for sul(II), which was absent in ditch water. It was noted that tet(W) and tet(O) were also present in treated drinking water and recycled wastewater, suggesting that these are potential pathways for the spread of ARGs to and from humans. On the basis of this study, there is a need for environmental scientists and engineers to help address the issue of the spread of ARGs in the environment.

Introduction

The spread of antibiotic-resistant pathogens is a growing problem in the U. S. and around the world. Recently a 2000 World Health Organization (WHO) report (*1*) focused on antibiotic resistance as one of the most critical human health challenges of the next century and heralded the need for "a global strategy to contain resistance". According to the report, more than two million Americans are infected each year with resistant pathogens and 14 000 die as a result. The rapid growth of the problem emphasizes the need for intervention. For example, vancomycin is currently considered to be the most powerful antibiotic of "last resort", yet within 10 years the incidence of vancomycin-resistant enterococci (VRE) increased in the United States from 0% to 25% (*2*, *3*). Resistance to penicillin, the antibiotic that originally revolutionized human health 50 years ago, is now as high as 79% in *Staphylococcus pneumoniae* isolates in South Africa (*4*, *5*). Alarmingly, diseases that were once considered to be eradicated, such as tuberculosis, are now beginning to make a comeback because of antimicrobial resistance (*1*, *6*, *7*). As with other dangerous pollutants that spread in the environment and threaten human health, there is a need for environmental scientists and engineers to help address the critical problem of microbial resistance to antibiotics.

The rise of antibiotic resistance is considered to be closely linked with the widespread use of antibiotic pharmaceuticals in humans and animals. In particular, more than one-half of the antibiotics used in the U. S. are administered to livestock for purposes of growth promotion or infection treatment (*8*, *9*). In both animals and humans, up to 95% of antibiotics can be excreted in an unaltered state (*10*, *11*). Some removal has been observed in wastewater treatment plants (WWTPs); however, as is true with the larger problem of pharmaceutical compounds, WWTPs are not designed for the removal of micropollutants (*12*−*14*). Residual antibiotics thus are released into the environment where they may exert selection pressure on microorganisms. While overprescribing or other improper use/disposal of antibiotics in humans is generally considered to contribute to the problem, several studies have also linked agricultural antibiotic use with antibiotic-resistant infections in humans (*15*−*23*). For example, avoparcin, an antibiotic growth-promoter used in poultry, was recently banned in Europe because of its association with the development of vancomycin-resistant enterococci (*24*).

Because of the direct selection pressure that antibiotics exert on organisms carrying antibiotic resistance genes (ARGs), the transport pathways of antibiotic-resistant microorganisms and the ARGs that they carry are expected to be similar to the pathways of antibiotic pharmaceuticals. In fact, it is likely that ARGs persist further in the pathway, considering that in many cases they are maintained in the microbial populations even after the antibiotic selection pressure has been removed (*25*−*28*). Also, horizontal gene transfer (HGT) is a major mechanism for sharing ARGs between microbes and has been documented to occur between nonpathogens, pathogens, and even distantly related organisms, such as Gram-positive and Gram-negative bacteria (*25*, *29*−*31*). In many cases, ARGs have been discovered to occur as part of multiple antibiotic resistant (MAR) superintegrons, which may contain over 100 ARG cassettes (*32*). These MAR superintegrons cause multiple-drug resistance in organisms, meaning that even when very different antibiotics are used, one antibiotic may coselect for resistance to other antibiotics (*5*, *33*). MAR gene cassettes and ARGs are notorious for being associated with plasmids and/or transposons that facilitate HGT. Finally, even if cells carrying ARGs have been killed, DNA released to the environment has been observed to persist, to be protected from DNAse, especially by certain soil/clay compositions, and to be eventually transformed into other cells (*34*−*36*). For all of these reasons, ARGs in and of themselves can be considered to be emerging "contaminants" for which mitigation strategies are needed to prevent their widespread dissemination.

The purpose of this study was to document the occurrence of tetracycline and sulfonamide ARGs in various environmental compartments in northern Colorado. These two ARG

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groups were chosen because sulfonamide and tetracycline antibiotics have been previously characterized in Poudre River sediments and shown to relate to urban/agricultural activity (*37*). The breadth of the study included Cache La Poudre (Poudre) River sediments, dairy lagoon water, irrigation ditch water, a wastewater recycling plant (WRP), and two drinking water treatment plants (DWTPs). The hypothesis was that environmental compartments most directly impacted by urban/agricultural activity would have significantly higher concentrations of ARGs than less impacted and pristine environments. Irrigation ditch waters, which were directly adjacent to farms, were investigated as a potential pathway of ARGs from farms to the Poudre River, while the WRP and the DWTPs were explored as potential routes of human environmental input and consumption. The presence/absence of several ribosomal protection factor tetracycline ARGs and folic acid pathway sulfonamide ARGs was determined using a polymerase chain reaction (PCR) detection assay, and four commonly occurring ARGs were further quantified by quantitative real-time PCR (Q-PCR). Documenting the baseline occurrence of ARGs in a cross-section of environmental compartments will take a step toward understanding and modeling the fate and transport phenomena associated with these emerging contaminants.

Experimental Section

Poudre River Sediment Sampling. Because of its pristine origins and zonation corresponding to land use, the Poudre River has served as a good model for relating human and agricultural activities with the occurrence of antibiotic pharmaceuticals (*37*) and ARGs (*38*). Five sampling sites were the focus of this study, numbered sequentially in the direction of flow from west to east, with the following characteristics: site 1, pristine location at the river origin in the Rocky Mountains; site 2, light-agriculture-influenced area; site 3, urban-influenced area at the outlet of the Fort Collins Drake WWTP; site 4, heavy-agriculture-influenced area between Fort Collins and Greeley; and site 5, heavy-agriculture- and urban-influenced area just east of Greeley, which is a major center for the meat-packing industry. Over 90 confined animal feeding operations (CAFOs), dairies, and ranches are located between sites 3 and 5. Further attributes of the Poudre River watershed that contribute to its suitability for investigating the impacts of urban and agricultural activity on antibiotics and ARGs have been described previously (*37*, *38*).

Sediment samples were collected along the Poudre River at the five sites on August 18, 2005, October 27, 2005, and February 17, 2006. The flow rates on these three dates were 1.04, 14.19, and 0.14 $m^3\ s^{-1}$, respectively (U. S. Geological Survey station number 06752260, Fort Collins, CO). Sampling at three points in time provided insight into potential temporal variations in ARG concentrations, and the February 17th date is exactly 1 year later than a previously published sampling date (*38*). 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.

Bulk Water Sampling. Irrigation ditch waters were investigated as a potential pathway of ARGs from farms to the Poudre River. Grab samples of bulk water were collected in sterile containers from irrigation ditches on August 18, 2005, corresponding to the August sampling date of the Poudre River sediments. All irrigation ditches were located between site 4 and site 5 on the Poudre River within a 3.5 km × 2 km zone north of the river, and a total of ten locations were sampled. To investigate a potential source of ARGs within this zone, a microaerophillic dairy lagoon (~1 mg/L dissolved oxygen in the upper 1 m) and an anaerobic dairy lagoon (0 mg/L dissolved oxygen) from an anonymous farm located 8 km from site 5 were sampled on October 20, 2005. Finally, source water, and pre-chlorinated, and post-chlorinated bulk water were collected from two anonymous DWTPs and an anonymous WRP in northern Colorado in February, 2005. The DWTP was studied as a potential direct route of ARGs to consumers, and the WRP was considered a potential human input into the environment. To collect fine particulates from the dilute ditch water, DWTP, and WRP samples for subsequent analysis, 500 mL of well-mixed sample was filtered using a 0.45 μm glass fiber filter (Whatman). This concentration step was not required for dairy lagoon samples.

DNA Extraction. DNA was extracted from 0.5 g of composited sediment using the FastDNA Spin Kit for Soil (MP Biomedicals) and from 1.8 mL of dairy lagoon water using the Ultraclean Microbial DNA Kit (MoBio Laboratories, Inc.) according to manufacturer protocol. Both approaches employ a bead-beating procedure. For fine particulates collected on filters from bulk water, the filters were cut into small pieces and added directly to the extraction tubes. Extraction yield and the quality of the DNA were verified by agarose gel electrophoresis and spectrophotometry.

Detection and Quantification of ARGs. Polymerase chain reaction detection assays were used for broad-scale screening of the presence/absence of five ribosomal protection factor tetracycline ARGs (tet(BP), tet(O), tet(S), tet(T), and tet(W)) (*39*) and four folic acid pathway sulfonamide ARGs (sul(I), sul(II), sul(III), and sul(A)). Development and validation of sul primers was described in Pei et al. (*38*). Positive controls consisted of cloned and sequenced PCR amplicons obtained from Poudre River sediments. Both positive and negative controls were included in every run, and negative signals were confirmed by spiking positive control template into the sample to verify a signal. Forty cycles were used to improve chances of product formation from low initial template concentrations. Further details on reaction mixes and temperature programs are available in Pei et al. (*38*); note that annealing temperatures for tet primers vary from Aminov et al. (*39*). Two tetracycline ARGs (tet(W) and tet(O)) and two sulfonamide ARGs (sul(I) and sul(II)) that were commonly occurring according to the PCR presence/absence assays were further quantified by Q-PCR using a SybrGreen approach. For further details on Q-PCR methods, see Pei et al. (*38*). Eubacterial 16S rRNA genes were quantified according to the TaqMan Q-PCR method described by Suzuki et al. (*40*) so that ARGs could be normalized to the total bacterial community. This provided a means to correct for potential variations in extraction efficiencies. By quantification of 16S rRNA genes, it was also possible to compare ARGs proportionally between samples of different overall population sizes. Matrix effects associated with extraction of DNA from environmental samples were corrected for by performing spiked matrix control tests and determining template suppression factors as described in Pei et al. (*38*). All Q-PCR analyses were performed using a Cepheid SmartCycler (Sunnyvale, CA).

Statistics. The influences of the environment (sites, ditch water, and dairy lagoons) on the normalized and non-normalized copies of ARGs were analyzed using the Mixed Procedure, which fits a variety of mixed linear models to data. This provides the flexibility of simultaneously modeling means, variances, and covariances (*41*−*44*). Through the use of this test, it was thus possible to comprehensively compare overall differences between different environmental compartments with respect to ARG concentrations. For comparison of the five Poudre River sites, multiple sampling time points were treated as replicates. Mixed Procedures were conducted using SAS 9.0 (SAS Institute Inc., Cary, NC). A

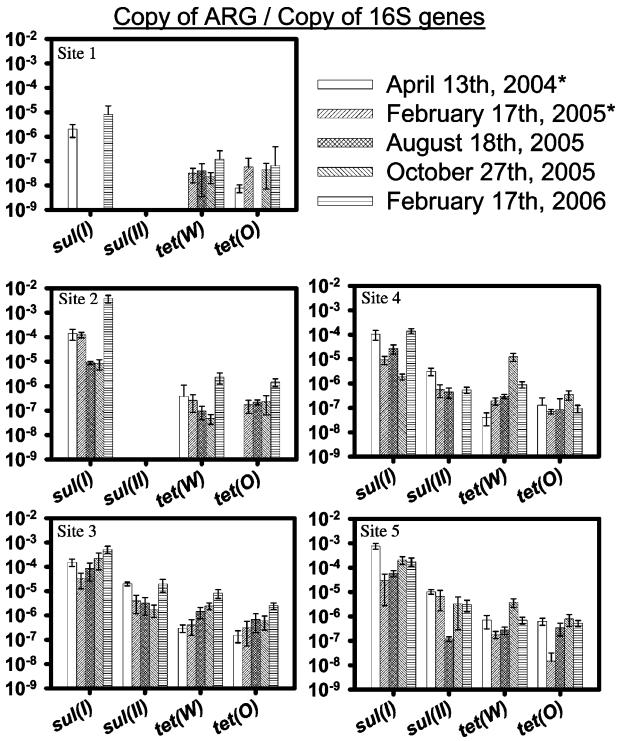

FIGURE 1. Distribution of four ARGs (sul(I), sul(II), tet(O), and tet-(W)) in Poudre River sediments on three sampling dates, compared to two previously published sampling dates (April 13, 2004, and February 17, 2005 (*38*)), as determined by Q-PCR: site 1, pristine site; site 2, light agricultural activity; site 3, heavy urban activity; site 4, heavy agricultural activity; site 5, heavy urban and agricultural activity. Error bars represent the standard deviation of six measurements from three independent Q-PCR runs analyzing DNA extract from composite samples.

p-value <0.05 was considered to indicate significance. Averages and standard deviations of all data were determined using Microsoft Excel, 2003.

Results and Discussion

Occurrence of ARGs in Northern Colorado. Figure 1 summarizes the Q-PCR data obtained for the four ARGs at the five Poudre River sites, while Figure 2 summarizes the same analyses for the ditch waters and dairy lagoon water. When August 2005 data for the Poudre River sediments are compared with the dairy lagoon and ditch water, the following trend is observed with respect to ARG concentrations: dairy lagoon water > ditch water > river sediments ($p < 0.0001$), for all ARGs except sul(II), which was absent from the ditch waters. This is based on pooling of all 10 ditch water sites, the two dairy lagoons, and sites 4 and 5, which were directly adjacent to the ditch water sampling locations. Within each of these three pools, there was no statistical difference observed among the samples. Therefore, it was observed as expected that environmental compartments most directly impacted by human/agricultural activity showed higher concentrations of ARGs. This trend is even stronger in considering absolute quantities of ARGs (not normalized to 16S rRNA genes), because the concentration of cells in the dairy lagoon water was orders of magnitude higher than that of the ditch water or the sediments.

In developing a hypothetical pathway for ARGs, a trend is not as clear. The overall trend in terms of ARG concentrations of dairy lagoon water > ditch water > river sediments suggests that on-farm compartments, such as lagoons may be the source of ARGs, which are subsequently attenuated in ditch water before reaching Poudre River sediments.

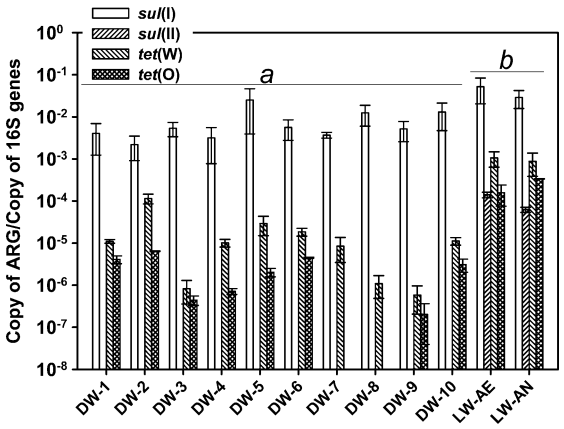

FIGURE 2. Distribution of four ARGs (sul(I), sul(II), tet(O), and tet-(W)) at 10 sampling points of irrigation ditch water (DW-1−DW-10) located between site 4 and site 5 compared with that of a microaerophillic dairy lagoon (LW-AE) and an anaerobic dairy lagoon (LW-AN). DW samples were concentrated from 500 mL, and LW samples were extracted directly from 1.8 mL. All samples were normalized to the total 16S rRNA genes. Error bars represent three independent Q-PCR runs in duplicate. The labels a and b indicate that the data sets fell into two statistically different groups, according to the Mixed Procedure.

However, this trend is not supported in terms of sul(II), which is entirely absent from the ditch water and therefore cannot be the source of what is observed in the Poudre River sediments. An alternative source of the sul(II) that appears at sites 4 and 5 could instead be human inputs. This is supported by the data presented in Figure 1, in which it is observed that sul(II) is consistently present at high levels on average at site 3, which is at the point of discharge of the Drake WWTP, while consistently lower (comparing each date sampled) at site 4 (entirely absent for the October event) and equivalent or lower at site 5, which has mixed human/agricultural inputs. Because sul(II) is present in the dairy lagoon waters, it must also have agricultural sources, but it may attenuate too quickly to be transported to the ditches and subsequently to the river sediments. On the basis of this study and a previous study (*38*), it is appears that of the four ARGs quantified sul(II) is the most sensitive indicator of human/agricultural impact, and thus it is suggested that it attenuates quickly in the absence of direct inputs. The other ARGs in the Poudre River sediments at sites 4 and 5 may be of either/both human and agricultural origin, since they followed a decreasing trend from the dairy lagoon through the ditch water but were also present at site 3.

In addition to having higher concentrations of three out of four of the ARGs, the dairy lagoon water was also observed to have more different kinds of ARGs present than the irrigation ditch water according to the PCR assay (Table 1). Together with the Q-PCR results, these data further support the concept that there is some attenuation of ARGs between any linkages that may connect dairy lagoon water and irrigation ditch water. Future work should implement ARG fingerprinting/source tracking to fully characterize the potential pathways.

Temporal Variations of ARG in Poudre River Sediments. As observed in a previous study that compared a high-flow sampling point (6.8 m^3 s^{-1}, April 2004) with a low-flow sampling point (0.6 m^3 s^{-1}, February 2005), the ARG concentrations in the Poudre River sediments are variable with time (*38*). To better understand temporal variations in ARG concentrations, the Poudre River sediments were sampled at three additional time points and compared with the two previously published time points. The February sampling point in this study took place exactly 1 year after

TABLE 1. PCR Presence/Absence Assay of Various ARGs in Ditch (DW)[a] and Dairy Lagoon (LW) Water[b]

ARG	DW-1	DW-2	DW-3	DW-4	DW-5	DW-6	DW-7	DW-8	DW-9	DW-10	LW-AE	LW-AN	+ control
tet(BP)	−	−	−	−	−	−	−	−	−	−	−	−	+
tet(O)	+	+	+	+	+	+	−	−	+	+	+	+	+
tet(S)	−	−	−	−	−	−	−	−	−	−	−	−	+
tet(T)	−	−	−	−	−	−	−	−	−	−	+	+	+
tet(W)	+	+	+	+	+	+	+	+	+	+	+	+	+
sul(I)	+	+	+	+	+	+	+	+	+	+	+	+	+
sul(II)	−	−	−	−	−	−	−	−	−	−	+	+	+
sul(III)	−	−	+	+	+	−	−	−	−	−	+	+	+
sul(A)	−	−	−	−	−	−	−	−	−	−	−	−	+

[a] Collected August 18, 2005. [b] Collected October 20, 2005.

the previous February event. In support of the relationship between ARG concentration and relative environment impact observed above, the pristine site (site 1) consistently had the lowest average concentrations of ARGs with time, with sul(II) completely absent and no individual ARG consistently present at all five sampling times (Figure 1). When presence/absence of ARGs are compared, site 2 appears to be the next lowest in terms of overall impacts. For example, sul(II) is consistently absent at site 2, and tet(O) was absent in one of the five sampling events, whereas these genes were consistently present at sites 3, 4, and 5. In terms of ARG concentrations, tet(W) and tet(O) at site 2 were equal or less than site 3; however, these two genes were sometimes higher and sometimes lower than at sites 4 and 5. On the basis of ARG averages and presence/absence of ARGs, sites 1 and 2 were the least impacted, as expected.

When the Mixed Procedure was applied to the data, in which the time points were pooled as replicates, it was found that there was no statistical difference between the five sites for the 16S normalized data, except in the case of sul(II) ($p = 0.0117$). However, when the same test was performed with non-normalized data, it was found that sites 1 and 2 were statistically lower than sites 3, 4, and 5 in terms of sul(I) ($p = 0.00296$), sul(II) ($p = 0.0199$), and tet(O) ($p = 0.0102$). Though normalizing to 16S genes provides a comparison of ARGs as a proportion of the total population, arguably it may be the absolute quantities of ARGs that are more critical.

While spatial variations in ARGs could be fairly well-characterized, it is difficult to identify clear temporal patterns. Comparison of the two February sampling dates that were exactly a year apart provides some insight. All four genes were either the same on average for both events (tet(O) for sites 1 and 4 and sul(II) for sites 4 and 5) or higher in the 2006 event (all other genes, except sul(II) at sites 1 and 2, where it was not present) (Figure 1). This suggests the possibility that all ARGs are increasing in concentration with time. However, the trends in between these two dates do not support this. Only tet(W) and tet(O) at site 3 increase consistently with time. All remaining ARGs at the five sites either decrease before increasing (e.g., tet(W) at site 2 and sul(II) at site 3), are constant and then increase (e.g., tet(O) at site 2 and tet(W) at site 1), or increase and then decrease (e.g., tet(W) at sites 4 and 5) (Figure 1). Therefore, no clear trend was identified with time.

It was also attempted to analyze trends in the data with respect to river flow rate. This was of interest because flow rate directly relates to runoff and nonpoint source inputs, which were hypothesized in the previous study to play a role in the observed increase in the number of kinds of ARGs detected in Poudre River sediments (*38*). The October 2005 sampling date provided a second sampling date at high flow (14.9 $m^3\ s^{-1}$), compared to the previously published April 2004 high-flow sampling date (6.8 $m^3\ s^{-1}$). (All other dates were at or below 1.0 $m^3\ s^{-1}$.) Interestingly, all four ARGs increased on average at site 5 in comparing the high-flow

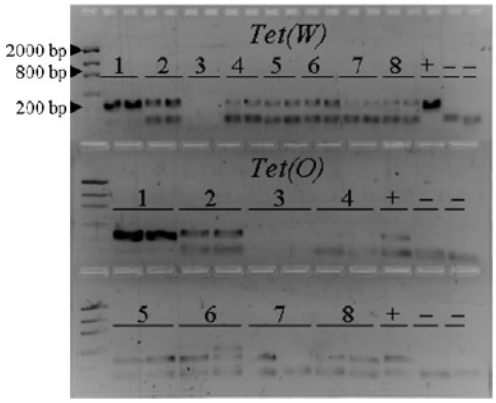

FIGURE 3. Agarose gel analysis of PCR presence/absence (in duplicate) of two ARG families, tet(W) and tet(O): + = positive control; − = negative control. The presence of a band at the same molecular weight as + indicates the presence of an ARG: 1 = WRP effluent; 2 = WRP chlorinated effluent; 3 = DWTP a influent; 4 = DWTP a treated water pre-chlorination; 5 = DWTP a treated water post-chlorination; 6 = DWTP b influent water; 7 = DWTP b treated water pre-chlorination; 8 = DWTP b treated water post-chlorination. The band appearing below 200 bp is consistent with a primer dimer.

October event with the immediately previous low-flow event in August (Figure 1). At site 4, tet(W) and tet(O) increased, but sul(II) stayed the same, and sul(I) decreased. There was no effect at all at site 3, which is affected primarily by point discharge rather than runoff, site 2, or site 1. However, attempts to plot ARG concentrations versus flow rate did not reveal any clear trend. Thus, it is still not possible to make a conclusive judgment on the effect of flow rate on ARG concentrations, though the role of nonpoint source inputs merits further investigation. To accomplish this, it would be necessary to gather more data with time/flow or monitor a much more controlled and smaller-scale system.

Wastewater Recycling Plant and Drinking Water Treatment Plants. A PCR presence/absence assay was conducted on the influent, intermediate effluent, and final effluent of two drinking water treatment plants (DWTP "a" and DWTP "b") and the pre-chlorinated and chlorinated effluent of a WRP. It was observed that both tet(W) and tet(O) were present at detectable levels in all samples except the source water for DWTP "a" (Figure 3). This indicates that the same two genes that were common in various environmental compartments in northern Colorado are also present in treated recycled wastewater and bulk drinking water. These two genes also showed a response to the level of impact; e.g., they were highest in dairy lagoon water and ditch water and lowest on average at the pristine site. On the basis of the intensity of the signal, they were also higher in the recycled wastewater than in the drinking water, as would be expected. Though these two ARGs are not directly associated with any known human pathogens, they may be indicators of links

between human/agricultural activity and ARGs in drinking water. Considering that drinking water is a direct route to human consumers, this emphasizes the need to better understand the pathways by which ARGs are spread in the environment and potential ways that the spread of ARGs may be reduced. For example, vancomycin resistance genes were found in drinking water biofilms in a recent study (*45*). Considering that vancomycin is typically the antibiotic of last resort when all else fails, this underscores the need to address this issue before it is too late. One possibility may be to make simple modifications to wastewater and drinking water treatment plants to reduce the spread of ARGs.

ARGs as Emerging Contaminants. On the basis of this study it is clear that ARGs are present in various environmental compartments, including river sediments, irrigation ditch water, dairy lagoon water, DWTPs, and a WRP. Furthermore, quantitative techniques incorporating Q-PCR provide a means to compare the concentrations of ARGs associated with the known urban and agricultural impacts, which provides a more direct measure than previous culture-based methods. On the basis of this occurrence survey, it is argued that ARGs are emerging contaminants that need to be further studied in the paradigm of environmental science and engineering. The concept of ARGs as "pollutants" has also been suggested by Rysz and Alvarez (*46*).

It should be noted that besides the tetracycline and sulfonamide ARGs that were the focus of this study, there are numerous other ARGs that have been described in the literature and likely even more that have not yet been discovered, each potentially with its own unique properties. Thus, each ARG may have different behaviors with respect to fate and transport and response to physical, chemical, and/or biological treatment. In terms of defining fate and transport characteristics of ARGs in general, it is expected that their behavior will be distinct in comparison to "typical" contaminants. For example, ARGs may be sequestered with bacteria, which are themselves transported, or they may be present as naked DNA bound to clay particles (*47*). Furthermore, ARGs may actually amplify in the environment under some conditions. This is indeed a unique contaminant property. Considering the significance of the problem of the spread of antibiotic resistance, further effort by environmental researchers to better understand these emerging contaminants is well-warranted. This is especially true as the rate of discovery and development of new antibiotics is continually declining (*48*), while the corresponding development and spread of resistance is occurring at a rapid pace. On the basis of this study, understanding ARGs as emerging contaminants can add a new and important angle to helping to approach this important problem.

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