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Agricultural contamination impacts antibiotic resistance gene abundances in river bed sediment temporally

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ABSTRACT

Kewaunee County, Wisconsin is an agricultural area dominated by concentrated animal feeding operations and manure fertilized cropland. The objective of this study was to characterize chemical and antibiotic resistance gene (ARG) profiles of 20 surface water locations in Kewaunee County to better understand relationships between agricultural contamination and ARG abundance over one year. Surface water (n = 101) and bed sediment (n = 93) were collected from 20 sites during five timepoints between July 2016 and May 2017. Samples were analyzed for six genes (*erm*(B), *tet*(W), *sul1*, *qnrA*, *intI1* and 16S rRNA) and water chemistry and pollution indicators. *qnrA*, *intI1* and *sul1* genes in surface water were significantly higher than *erm*(B) and *tet*(W); however, no difference was present in sediment samples. Redundancy analysis identified positive correlations of nitrate, *Escherichia coli*, and coliforms with *tet*(W) and *intI1* genes in sediment and *intI1*, *sul1* and *tet*(W) genes in water. Temporal patterns of ARG abundance were identified with significantly higher gene abundances found in sediment during Kewaunee County's manure fertilization period; however, surface water patterns were not distinct. Together, these results suggest Kewaunee County sediments serve as a site of accumulation for non-point source agricultural pollution and ARGs on a temporal scale associated with manure fertilization.

Keywords

antibiotic resistance, runoff, surface water, agriculture, manure fertilization; sediment

INTRODUCTION

The discovery and subsequent industrial development of antibiotics revolutionized healthcare. However, despite the efficacy of antibiotics, overuse and misuse of these compounds has led to an increase in bacterial antibiotic resistance in both the clinic and environment (Davies and Davies 2010; Berendonk *et al*. 2015). Antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs) are now being recognized as emerging contaminants, and characterizing environmental reservoirs of these contaminants is necessary to better understand what impact they might have on human health (Pruden *et al*. 2006; Marti, Jofre and Balcazar 2013; Berendonk *et al*. 2015; Munita and Arias 2016). ARGs are a natural component of many environmental bacteria genomes and serve as a defense mechanism against antibiotics. However, increased anthropogenic activity has significantly altered the presence and abundance of ARGs in a variety of commensal and pathogenic bacteria, especially in regions with high antibiotic input such as wastewater treatment plants, healthcare and agriculture (Baquero, Martinez and Canton 2008; Allen *et al*. 2010; Heuer, Schmitt and Smalla 2011; Rieke *et al*. 2018).

Agriculture is frequently implicated as a source of excessive antibiotic use which promotes selection of ARGs since more than 80% of all antibiotics produced in the United States are used on livestock farms (Hollis and Ahmed 2013). Antibiotic use in livestock selects for ARB within the animal gut and promotes the dissemination of ARGs into the environment from manure, increasing the likelihood that resistant bacteria (and associated genes) will enter soils, surface waters and groundwaters (Pan *et al*. 2011; Udikovic-Kolic *et al*. 2014; Wichmann *et al*. 2014; Chambers *et al*. 2015). Additionally, it has been reported that between 25% and 75% of veterinary antibiotics are discarded unaltered in urine or feces which would provide a direct environmental selection pressure (Sarmah, Meyer and Boxall 2006). Although measurable antibiotic concentrations in the environment are typically low, recent evidence indicates that resistant bacteria are positively selected even at

very low concentrations (Gullberg *et al*. 2011). In these ways, both antibiotics and ARGs can enter the environment, proliferate through horizontal gene transfer in environmental bacteria, and disseminate beyond livestock feeding areas (Peak *et al*. 2007; Noyes *et al*. 2016; Zhou *et al*. 2016).

Using manure as cropland fertilizer enhances the movement of ARB and ARGs as runoff events from fertilized agricultural fields have the potential to transfer contaminants from manure to surface water systems (Halling-Sørensen *et al*. 1998; Stoll *et al*. 2012; Rieke *et al*. 2018). These runoff events create non-point sources of pollution, making mitigation strategies difficult. Additionally, seasonal factors such as precipitation, nutrient availability and stream velocity impact the movement and abundance of contaminants such as ARGs and antibiotic residues in surface waters (Knapp *et al*. 2012). Characterizing the impact of agricultural non-point source pollution on ARG dissemination and abundance in surface water ecosystems is useful for monitoring how large-scale farming impacts environmental reservoirs of antibiotic resistance.

In this study, we aimed to understand the relationship between manure fertilization, pollutant concentration and ARG abundance by using a combination of chemical, microbiological, and molecular techniques to characterize surface water ecosystems in Kewaunee County, WI. Kewaunee County is an agricultural region dominated by concentrated animal feeding operations (CAFOs), or farms that confine more than 1000 animal units on site for a minimum of 45 days. Farmers in Kewaunee primarily manage manure waste through seasonal cropland fertilization. Kewaunee County citizens are concerned that excessive manure fertilization is contaminating area groundwater, their primary drinking water source. A 2017 study found that 79 of 131 tested wells in Kewaunee County contained bacteria. Of those, 40 were contaminated with fecal bacteria of cattle origin, but the remaining 39 were contaminated with bacteria of an unknown or human origin (Borchardt *et al*.2017). As such, the primary source of contamination in Kewaunee County waters remains unclear, and little is known about the abundance of emerging contaminants, such as ARGs. We hypothesized that ARG abundance in Kewaunee County surface waters is influenced by CAFO farming and manure fertilization contamination. A county ordinance restricts application of manure to cropland during the frozen ground period of January 1–April 15th, and as such we predicted increases in contaminant load and ARG abundance in surface water systems on a temporal scale associated with the agricultural growing season. Differences in individual ARG abundances are expected with genes encoding resistance to commonly used agricultural antibiotics predicted to be highest in abundance. Results from this study will provide a better understanding of the impact of agricultural practices on surface water ecosystems and provide data for manure management policy decisions in Kewaunee County.

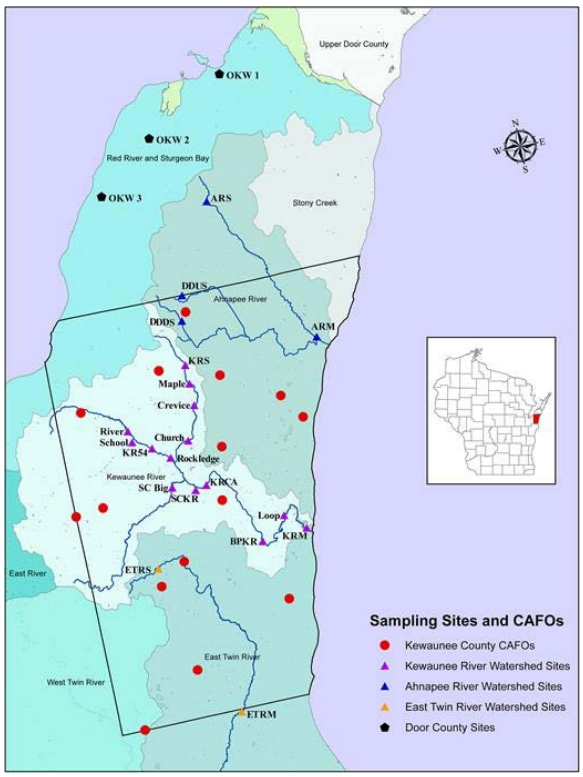
MATERIALS AND METHODS

Site description and data collection

Kewaunee County, Wisconsin is an agricultural region with 175 449 acres of farmland comprising nearly 100 000 cattle (Census of Agriculture 2012). As of 2015, the total human population of Kewaunee County was just over 20 000 individuals (nine individuals/km^2), meaning cattle outnumber humans 5:1. Three primary surface water systems are located in Kewaunee County: the Ahnapee River in the north, the Kewaunee River in the middle and the East Twin River in the south. These three rivers and their corresponding watersheds encompass one beef and 14 dairy CAFOs present in Kewaunee County and as such are useful for evaluating the impact of large-scale farming on the dissemination and abundance of ARGs (Fig. 1, Kewaunee County CAFOs). Originally, 20 ‘high impact’ sites (sites within watersheds comprising CAFO farms) were chosen for seasonal monitoring at public access locations along the three listed rivers (Fig. 1, Table S1, Supporting Information; Kewaunee River Watershed Sites, Ahnapee River Watershed Sites, East Twin River Watershed Sites). Three additional ‘low impact’ sites in Door County, Wisconsin were selected to ascertain ARG abundance at sites with agricultural impact from small farms only (no CAFO impact) and similar geologies (Fig. 1, Door County Sites). Two manure samples from Kewaunee County were also collected in May 2017. Dissolved oxygen and pH were measured in

water samples in the field (Mettler Toledo pH/ion Meter). Surface water (in 1 L acid-washed amber bottles) and grab sediment samples (transferred to Fisherbrand sterile sampling bags) were collected at one sampling timepoint each during July, September and October 2016 and February and May 2017. Samples were transported on ice and stored at 4°C for up to one week. Surface water samples were vacuum filtered in 150–250 mL aliquots through 0.22 µM mixed cellulose esters membrane filters (47 mm diameter, type GSWP, Millipore, Bedford, MA). Sediment samples were homogenized after removing debris and pebbles and subsampled directly. Replicate filters and sediment samples were stored at −20°C until DNA extraction.

Figure 1.



Sampling sites and concentrated animal feeding operations (CAFOs) within Kewaunee and Door counties, Wisconsin. Sampling sites are denoted by symbols differentiating their watersheds: Kewaunee River Watershed ( ), Ahnapee River Watershed ( ), East Twin River Watershed ( ) and Door County sites ( ). CAFO locations are denoted by red circles ( ) and were locationally identified via public permit access through the Wisconsin Department of Natural Resources.

DNA extraction and quantitative-PCR

DNA was extracted from 0.5 g of sediment using the DNeasy PowerSoil Kit (Qiagen) according to manufacturer instructions and from water using an in-house protocol as described previously for groundwater (Hristova, Lutenegger and Scow 2001) with the following modifications: Following liquid nitrogen freezing, tubes containing filters were vortexed for 20 s and incubated in a dry bath (80°C) for 5 min. Samples were then cooled on ice and centrifuged for 10 min at 20 000 x *g*. A 3 µL volume of 50 mg/mL RNAse A was added, and samples were then incubated for 30 min at 37°C followed by a 5 min cooling on ice. Next, 10 µL of 10% sodium dodecyl sulfate solution was added followed by 120 µL of Protein Precipitation solution (Promega). Samples were again incubated on ice for 5 min followed by a 10 min, 20 000 x *g* centrifugation step. Sample supernatants were then transferred to a microconcentrator column (Vivaspin 500; Sartorius), washed with TE and reduced to a final

volume of 50 µL. SYBR Green chemistry was used for all qPCR assays. Genes associated with antibiotic resistance were selected for this study on the basis of: (1) genes conferring resistance to antibiotics frequently used in agriculture (e.g. tetracycline resistance gene *tet*(W), macrolide resistance gene *erm*(B)); (2) genes associated with mobile genetic elements or gene cassettes (integron-integrase *intI1*, sulfonamide resistance gene *sul1*); (3) genes conferring resistance to clinically important antibiotics with limited use in agriculture (eg: fluoroquinolone resistance gene *qnrA*). In total, six genes were quantified: ARGs *tet*(W), *erm*(B), *sul1* and *qnrA*; clinical Class 1 integron-integrase gene *intI1*, and 16S rRNA genes as a proxy for total bacteria.

All genes were quantified in duplicate on a Real-Time PCR System (Bio Rad CFX Connect) from two separate DNA extractions per sample and averaged for total gene abundance (four reactions total per sample). Primers and cycling conditions used in this study are listed in Table S2 (Supporting Information). qPCR reaction mixtures contained 15 uL of Master Mix (10 uL SYBR Green MM, 2 uL of 10 nM each F/R primers, 3 uL H_2O) and 5 uL of 4 ng/uL gDNA. Standard curves were generated by cloning full length, amplified antibiotic resistance genes into a pGEM T-Easy vector (Promega) and confirming insertions via Sanger sequencing. Seven-point standard curves and negative template controls were present in duplicate for each PCR run. Limits of quantification for each gene were as follows: *erm*(B) = 15 copies, *tet*(W) = 11 copies, *sul1* = 41 copies, *qnrA* = 120 copies and *intI1* = 42 copies per 1 g of sediment or 100 mL of surface water. Quality control for qPCR reactions included optimizing the gDNA dilution necessary to minimize inhibition from co-extracted contaminants, melting-curve analysis for each run, and reamplification of duplicate samples containing more than 0.5 Ct value variance.

Measurement of additional environmental variables

Nitrate and phosphate concentrations within unaltered surface water samples were measured using Ion Chromatography (Dionex ICS-1100, Thermo Scientific). Pharmaceuticals, personal care products and hormones within collected surface water samples were extracted by solid phase extraction methods and analyzed by liquid chromatography tandem mass spectrometry (LC/MS/MS) as described in detail elsewhere (Dodgen *et al*. 2017). Metal concentrations in 0.5 g dry-weight sediment samples (May 2017 only) were measured using inductively coupled plasma mass spectrometry (ICP-MS) after digestion with concentrated nitric acid. Fecal indicators, coliform bacteria and *Escherichia coli* present in 100 mL of unaltered water sample were measured within 24 h of sample collection using the USEPA approved Colilert Quanti-Tray®/2000 Method (USEPA 2010).

Geospatial analysis

For each Kewaunee River Watershed Site (Fig. 1), the total watershed drainage area was calculated by first downloading a 30 m digital elevation model (DEM) from the national elevation data set (NED) of the US Geological Survey. Next, the watershed boundaries were delineated by using the hydrology toolbox in ArcGIS version 10.4 (ESRI Inc., Redlands, California) and the total acreage area draining into each site was calculated. Direct, linear overland distance to nearest upstream CAFO was calculated using the drawing tool Google Earth Pro with reference to the delineated watersheds.

Statistical analyses

Data manipulation and statistical analyses were completed in PRIMER-E version 7 with the PERMANOVA+ add on package and R version 3.3.2. Data were fourth root transformed for use in all statistical analyses to stabilize variance from multiple low or zero values (Parravicini *et al*. 2010). No detection values of measured genes were calculated by using the standard formula LOD/2 (Armbruster and Pry 2008; Bustin *et al*. 2009). Using R software, missing data were imputed using the predictive mean matching method within the package *mice*. Redundancy Analysis, Student's t-test, and ANOVA were also completed in R. PRIMER-E (v7) with the PERMANOVA+ add on package was used to compute clustering analysis to assess similarity between samples (Clarke and Gorley 2015). For this purpose, a resemblance Bray–Curtis similarity matrix was constructed. Cluster and SIMPROF analysis were used to study the true structure within the data. The SIMPROF analysis examined pairs of samples in each

cluster, where observed similarity (non-significant difference) is larger than expected by chance (red branches) with parameters of 999 permutation and 5% significance. The PERMANOVA+ add on package was used to test for differences in ARG and 16S rRNA gene abundances based on time, phase and location factors followed by *post-hoc* t-tests to determine pairwise differences. Following Clarke and Gorley (2015), permuted calculations of *P* were used when unique permutation values were >100 and Monte Carlo calculations of *P* were used when unique permutations were <100.

RESULTS

Abundance of pollutants and ARGs in Kewaunee County surface waters and sediments

Surface water (*n* = 101) and bed sediment (*n* = 93) were sampled at a total of 20 sites impacted by CAFO farming and manure fertilized cropland ('high impact') during five time points in 2016–2017 and three sites with no impact from CAFO farming ('low impact') in May 2017 only. Sampling sites were predominately located on the Kewaunee River (*n* = 14), but also included the Ahnapee River (*n* = 4), East Twin River (*n* = 2) and Door County sites (*n* = 3). Additional information for all sites including watershed descriptions and geographic locations is available in Table S1 (Supporting Information).

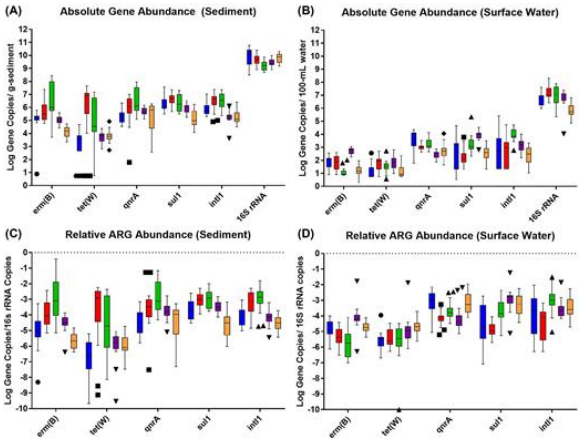
Five antibiotic and antimicrobials, seven hormones and eight personal care products and pharmaceuticals were measured in surface water samples. Of the five measured antibiotic and antimicrobial agents, only two were detected at more than one timepoint (triclocarban and triclosan) and these were at low concentrations (all <35 ng/L, most <5 ng/L). This result was not unexpected as environmental water samples typically contain minimal concentrations of antibiotic compounds (Aga *et al*. 2016; Ebele, Abou-Elwafa Abdallah and Harrad 2017). Personal care products and hormones that were detected during at least two sampling dates included caffeine, fluoxetine (an antidepressant) and the hormone esterone-3-sulfate; however, pharmaceuticals acetaminophen, ibuprofen, carbamazepine, gemfibrozil, naproxen and the hormone 17β-estradiol were also detected during at least one sampling period. No personal care products or horm ones were present at all sampling sites except caffeine.

Additional nutrient and contaminant measurements included nitrate ions (N), phosphate ions (P), coliform bacteria, *E. coli* and metals. Of these, N and P (two indicators of nutrient load) were consistently present at concentrations above the USEPA limit for drinking water for N (10 mg/L; USEPA 2009) and the USEPA and WI EPA recommendation for P in surface water (0.05 mg/L; USEPA 1988). *Escherichiacoli* were also consistently present at concentrations above the EPA recommendation for recreational water (126 CFU/100 mL; USEPA 2012). Fecal contamination indicators (coliform bacteria and *E. coli*) were present at all sampling sites for all measured timepoints. Although metals were measured in May 2017 sediment samples only, elevated concentrations (>300 ppm) of Mg and Ca (background levels in surface waters in Wisconsin are 12 and 8 ppm, respectively; Lillie and Mason 1983) were present at multiple locations in the Kewaunee County watersheds.

Median absolute (normalized per g wet sediment or 100 mL water) and median relative abundance (normalized per 16S rRNA gene copies per g wet sediment or 100 mL water) of each gene measured within water and sediment samples of Kewaunee, Ahnapee and East Twin River watersheds are displayed in Fig. 2 (*n* = 20 per timepoint). Absolute abundance of 16S rRNA gene copies in the 20 'high impact' sites ranged from log 8.48 to 10.76 per gram wet sediment and log 5.58 to 8.32 in 100 mL surface water, and absolute abundance of ARGs and *intI1* (excluding outliers) ranged from log 3.53 to 6.59 per gram wet sediment and log 0.89 to 3.67 in 100 mL surface water (Tables S3 and S4, Supporting Information). In the three 'low impact' site samples collected in May 2017 only, absolute abundance for the 16S rRNA gene copies ranged from log 8.90 to 9.53 and log 5.40 to 6.76 per gram wet sediment and 100 mL surface water, respectively, while ARGs and *intI1* copy numbers ranged from log 3.34 to 4.65 and log 0.74 to 3.97 per gram wet sediment and 100 mL surface water, respectively. On an

individual gene basis, the highest mean relative abundance for all ARGs and *intI1* in sediment were measured in September 2016 and October 2016, while the highest mean abundance of 16S rRNA genes in sediment was measured in July 2016 and May 2017. In surface water samples, the highest mean relative abundance was more variable; September 2016 and February 2017 contained the highest values for *erm*(B) and *tet*(W), July 2016 and May 2017 for *qnrA*, and July 2016, October 2016 and February 2017 for *sul1*, while the highest abundance of 16S rRNA genes in surface water was measured in September 2016 and October 2016. On the year-long scale, no significant differences were found between individual gene abundance in sediment; however, *qnrA, sul1* and *intI1* gene abundances in surface water were significantly higher than *erm*(B) and *tet*(W) (ANOVA, post-hoc t-test, $P < 0.01$). Relative abundance of ARGs and *intI1* in the three 'low impact' site samples were not significantly different than the 'high impact' sites in May 2017 samples (Student's t-test, $P > 0.05$).

Figure 2.



Distribution of absolute (per gram wet sediment or 100-mL surface water) and relative (normalized per 16S rRNA gene) gene abundances. Log-transformed gene copies are shown for 20 sites during five sampling timepoints: July 2016 (■), September 2016 (■), October 2016 (■), February 2017 (■), and May 2017 (■) for (**A**) absolute abundance of bed sediment, (**B**) absolute abundance of surface water, (**C**) relative abundance of bed sediment, and (**D**) relative abundance of surface water. Tukey-style whiskers are shown for all genes with sample size $n = 100$.

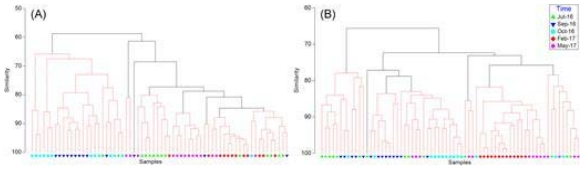
Manure samples, also collected in May 2017 only, contained 16S rRNA gene copies with a mean of log 10.44 and ARGs and *intI1* with a range of log 4.95–8.38 gene copies per gram of manure. The relative abundance of ARGs in sediment and water were significantly different (Student's t-test, $P < 0.001$) with sediment samples containing, on average, two orders of magnitude higher gene copies of ARGs. In the manure samples, the mean relative abundance of ARGs was higher than in sediment samples for the following genes: *erm*(B) (1000 fold higher), *tet*(W) (10 fold higher), *sul1* (100 fold higher) and *intI1* (100 fold higher). However, *qnrA* gene abundance was 10-fold higher in sediment samples than manure samples.

Temporal differences of ARG abundance are present in surface water and sediment

Absolute and relative ARG values displayed in Fig. 2 suggest that patterns in ARG abundance are present in Kewaunee County sediment and surface water. To identify patterns in gene abundance, the relative ARG abundance of Kewaunee River watershed sites ($n = 14$) was analyzed using hierarchical clustering analysis. A strong temporal separation in both sediment and water samples was observed with July, September and October sampling sites forming one cluster and February and May sampling sites forming another (Fig. 3A and B). This result suggests that ARG abundance varies temporally in the Kewaunee River, and the

observed clustering corroborates with the highest abundances in sediment (Table S3, Supporting Information) found during the manure application period (July, September and October samples) and the highest abundances in water (Table S3, Supporting Information) found during the frozen period (February) or immediately after the start of Kewaunee County's manure application ordinance (May). Data were additionally analyzed using PERMANOVA on the basis of location (spatial), time (temporal) and phase (environmental matrix) factors to determine if any significant relationships within or between factors were present. PERMANOVA results indicated that time and phase differences were significant ($P = 0.0001$). Spatial differences, while significant in combination with the other factors, were minimal upon further analysis. In sediment samples, the relative ARG abundances of all sampling months were significantly different from one another ($P < 0.01$) with the exception of July 2016 and February 2017 samples. In surface water, only July 2016 and May 2017 relative ARG abundances were significantly different from all other months ($P < 0.01$). Together these analyses suggest that temporal patterns are present in the ARG composition of both sediment and surface water samples from Kewaunee County.

Figure 3.



Hierarchical clustering analysis with group average linking based on Bray–Curtis resemblance matrix of fourth-root transformed ARGs copies (relative abundance) in (**A**) sediment samples and (**B**) surface water samples of the Kewaunee River watershed ($n = 14$) during five sampling timepoints between July 2016 and May 2017. The SIMPROF analysis examined pairs of samples in each cluster, where observed similarity (non-significant differences) is larger than expected by change (true differences, red branches) indicating true temporal differences between early and late fertilization season and ARG abundance.

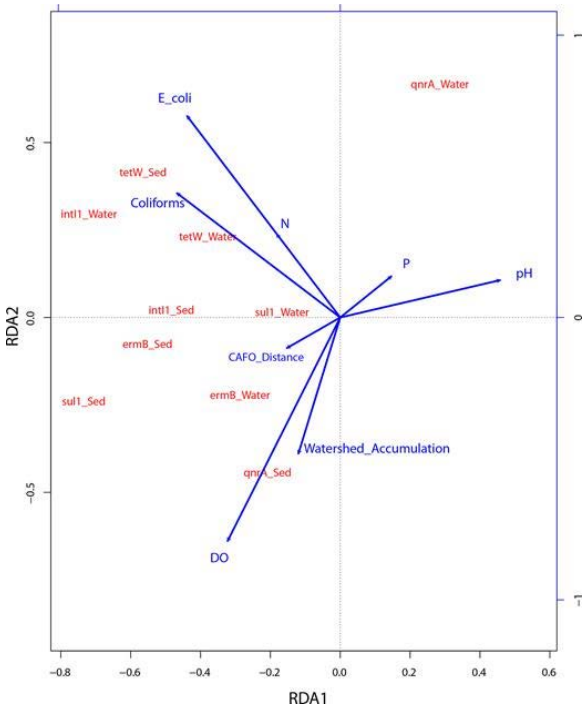
To determine if observed temporal differences in the ARG abundance of Kewaunee River sampling sites were due to true differences in ARG abundance or only due to seasonal changes in bacterial populations, we performed PERMANOVA on absolute ARG abundances normalized to either 1 g of sediment or 100 mL of surface water ($n = 20$ per site). The temporal differences identified in the clustering analysis remained statistically significant (PERMANOVA; $P = 0.001$) when all 20 'high impact' sites were included, regardless of normalization method. However, differences in sampling month containing the highest mean abundance of individual ARGs were identified in surface water samples based upon the normalization method. For example, mean relative abundances of all ARGs in surface water were found to be the highest in May 2017 samples (in addition to a variety of other months based upon individual gene), but mean absolute abundances in May 2017 were only the highest for *qnrA*. In sediment samples, the highest mean relative and absolute abundance of ARGs were both found in September 2016 and October 2016. These results suggest that the pattern of high ARG abundance in the summer and fall months and low ARG abundance in the winter and spring months is a real trend in Kewaunee sediments; however, surface waters have a present but less defined pattern.

Environmental factors influence ARG distribution

In addition to identifying trends in ARG abundance associated with the Kewaunee County manure application ordinance, underlying correlations between environmental factors and ARG distribution were analyzed. In this study, factors chosen were either seasonal variables or site characteristic variables and included: nitrate ions (N), phosphate ions (P), *E. coli*, coliforms, overland distance to nearest upstream CAFO (CAFO distance), total watershed accumulation land area (Watershed Accumulation), dissolved oxygen (DO) and pH. Redundancy

analysis was used to analyze the effect of these eight factors on the five measured genes in both sampling matrices (Fig. 4). Permutation analysis of the model determined that the relationship between the eight factors and five ARGs was significant (P = 0.003). The resulting RDA plot indicated three major axes of correlated variables: Axis 1 consisting of a positive correlation with *E. coli*, coliforms and nitrate ions; Axis 2 consisting of a positive correlation with phosphate ions and pH and a negative correlation of these two variables with distance to upstream CAFO; and Axis 3 consisting of a positive correlation between dissolved oxygen and total watershed accumulation area. Genes *intI1* in water and *tet*(W) in both matrices were positively correlated with Axis 1 environmental variables. Genes *sul1* (both matrices), *erm*(B) (both matrices) and *intI1* in sediment were positively correlated with the distance to upstream CAFO. *qnrA* genes in sediment and water matrices were negatively correlated with one another, with *qnrA* genes in sediment positively correlated with Axis 3 and *qnrA* genes in water positively correlated with phosphate ions and pH.

Figure 4.



Redundancy analysis (RDA) biplot on fourth root transformed data for the quantitative correlation between environmental and site specific variables (explanatory) and the distribution of ARGs in sediment and surface water samples (response). Explanatory variables are represented as vectors while response variables are represented as points. Type II scaling was utilized to determine the correlations between explanatory and response variables.

To further explore the temporal trends of ARGs identified in clustering and PERMANOVA analyses, an RDA that included sampling month as an explanatory variable was also performed (data not shown). ARGs in sediment were positively correlated primarily with distance to upstream CAFO, dissolved oxygen, and September and October 2016 samples. This result supports previous sediment temporal trend conclusions in which the highest ARG abundances in sediment were found in September and October 2016 samples.

DISCUSSION

In this study, our goal was to characterize the chemical and ARG profiles of 20 surface water locations in Kewaunee County to better understand the relationship between agricultural contamination and ARG

abundance on a year-long scale. We identified multiple contaminants including nitrates, phosphates, *E. coli* and coliforms at most sites during all seasons which strongly suggests non-point source agricultural pollution is impacting surface waters in Kewaunee County. Additionally, a positive correlation between distance to nearest upstream CAFO location and multiple ARGs was identified, indicating that as the distance to the CAFO increases, so do the ARG abundance values, while a negative correlation was found between phosphate ions with distance to upstream CAFO and multiple ARGs (Fig. 4). This result also supports non-point source pollution via cropland manure spreading as the major source of contamination in Kewaunee County rather than a point source (or factors associated with point sources such as phosphates) from individual CAFO farm locations. Significant differences in individual gene abundance were present in surface water samples with *qnrA, sul1* and *intI1* genes found in significantly higher abundance than *erm*(B) and *tet*(W). Although no significant differences were present between individual genes in sediment, all measured gene abundances were significantly higher in sediment than surface water samples. Our results also suggest that temporal differences in ARG abundance are present and are linked to seasonal agricultural contamination associated with the manure application ordinance. Samples collected during the specified manure application period in Kewaunee County contained, on average, higher ARG copy numbers (particularly in sediment) regardless of normalization method (absolute or relative, Figs 3 and 4) and these differences were statistically significant (PERMANOVA).

Antibiotic resistance genes were present at most locations during all seasons. No significant differences were identified in gene abundance between 'high' and 'low' impact sites; however, this is likely a limitation of sampling the 'low impact' sites during one timepoint only. Relative gene abundances for *sul1* (10^{-5}–10^{-2} gene copies/16S rRNA copies) were similar to ranges found in other surface water ecosystems impacted by agriculture (Pruden *et al*. 2006; McKinney *et al*. 2010). *intI1* gene abundances have traditionally been measured in wastewater treatment plants, soils, or manure holding lagoons, but we did find that our measured values were similar to other studies of contaminated sites (Li *et al*2017; Peng *et al*. 2017). Although *tet*(W) values were typically lower than other measured genes in this study (10^{-7}–10^{-4} gene copies/16S rRNA copies), similar results were reported by Pruden *et al*. (2006, 2012) in the presence of measurable tetracycline in surface water. Notably, September and October 2016 sediments contained *tet*(W) copy values in the range of 10^{-7}–10^{-4} gene copies/16S rRNA copies, 10–100 fold higher than other contaminated sediments and 100–1000 fold higher than reported background values in sediments (Pruden *et al*. 2006). Additionally, *tet*(W) genes in sediment and surface water correlated strongly with measured coliforms, an indicator of fecal pollution (Fig. 4). *erm*(B) gene abundances were also similar to other agriculturally impacted surface water systems (Khan *et al*. 2013; Rieke *et al*. 2018). These results indicate that Kewaunee County surface water ecosystems contain elevated ARG abundances similar to or even higher than other studies monitoring the impact of agricultural contamination on surface waters.

Interestingly, we found that *qnrA*, *sul1* and *intI1* gene abundances in surface water were consistently 10–100 fold higher than *erm*(B) and *tet*(W) abundances. The most significant of these findings was the elevated abundance of *qnrA*, as this gene encodes resistance to fluoroquinolones, a class of antibiotics important for human health. Both *erm*(B) and *tet*(W) encode resistance for two more common classes of antibiotics used in livestock (macrolides and tetracyclines) and were expected to be elevated throughout samples, while *qnrA* was expected to be minimally detected due to limited reported use of fluoroquinolone drugs in food-producing animals (US-FDA 2018, USDA 2018). However, other studies have found similar results. Yang *et al*. (2017) found that *qnrA* genes were detected more frequently than *tet*(W) genes in freshwater lakes in China, and Cummings *et al*. (2011) found values similar to this study of *qnrA* genes in freshwater sediments (10^{-5}–10^{-2} copies *qnrA*/16S rRNA). The primers used for this study are able to detect both chromosomal *qnrA* genes and plasmid borne *qnrA* genes (Cummings *et al*. 2011). Sequencing of clones containing *qnrA* inserts from our samples indicated that most of these genes were plasmid derived (data not shown); thus, elevated *qnrA* genes could stem from multiple sources including non-point source pollution from human origin such as septic tank

leakage or mixed human and animal waste collection in lagoons, misuse of a class of limited access antibiotics, co-selection or differences in natural degradation rates of resistance determinants. Interestingly, *qnrA* genes in water samples were found to be negatively correlated with *qnrA* genes in sediment samples in the RDA; thus, additional study is necessary to determine specifically where these resistance genes are originating. Although *sul1* and *intI1* genes were also significantly higher in surface water samples than *erm*(B) and *tet*(W), this result is not as surprising. *intI1* has been identified in multiple regions of anthropogenic pollution and *sul1* has been correlated with upstream animal feeding operations (Pruden, Arabi and Storteboom 2012). Additionally, these two genes are commonly found co-located together on mobile genetic elements (Gillings *et al*. 2015). It is important to note that this trend was only found in surface water samples; no significant differences were found in gene abundance of sediment samples.

Patterns of ARG abundance throughout the one-year sampling event were of particular interest to us as this has not been well characterized. We tested for time, location and phase differences in ARG abundance in the 'high impact' sampling sites and found significant differences in both time and phase. Sediment samples were found to contain the highest abundance of all measured ARGs in the summer and fall sampling events, regardless of normalization method. It is unlikely that this trend is due to seasonal shifts in the total bacterial community as 16S rRNA genes were lowest in abundance in fall samples (Fig. 2). Sediment is considered an ideal site for contaminant accumulation and adsorption (Hu, He and Zhou 2012; Devarajan *et al*. 2015), and this likely plays a role in the higher gene abundances found in sediments in the summer and fall. Continuous contaminant inputs from manure fertilization and runoff contribute to a buildup of pollutants in sediments which can include ARGs and promote gene transfer and co-selection mechanisms contributing to the seasonality found in Kewaunee County sediments. Water samples were more variable with each season containing the highest abundance of at least one measured gene and additional differences when comparing absolute or relative abundance. Although ARG abundance in surface water showed significant temporal differences, no clear trend was present for absolute or relative abundances of ARGs nor for the 16s rRNA gene abundance in surface water. This contrasts with the well-defined seasonal pattern of ARGs in Kewaunee County sediments; however, it can likely be explained by the sampling method. Water samples were collected from an individual location at one timepoint rather than multiple timepoints over the course of 24 h or multiple days within one season. Collecting additional samples over a shorter duration is necessary to better understand temporal patterns in surface water samples.

This study highlights the importance of identifying temporal patterns of ARG abundances and environmental contaminants present in surface water ecosystems. Manure fertilization of cropland, from antibiotic treated or untreated animals, creates non-point source pollution that impacts nutrient, bacterial and contaminant loads which in turn can either directly increase or co-select for the increase of ARGs in the environment. Manure fertilization primarily takes place during the crop growing season (April to October in the Midwest United States) and seasonal differences in precipitation, stream flow and nutrient availability may differentially impact surface waters based upon time of application. Seasonal differences require individualized contaminant mitigation strategies which can include altering the manure fertilization period to reduce spreading during high stream flow and precipitation seasons, reducing runoff through alternative strategies such as cropland buffers or cover crops, and long-term seasonal monitoring of ARGs and contaminants. This study provides a better understanding of the presence, abundance and temporal patterns of ARGs and contaminants in a region impacted by CAFOs and manure fertilized cropland; however, additional data collection extending out over multiple years is necessary to confirm the observed temporal trends. Our data also serves as a resource for Kewaunee citizens and farmers to evaluate their current manure application policies for both environmental and human health. Additional studies are necessary to determine the microbial community and resistome composition which will provide information about the presence of bacterial pathogens and clinically important ARGs that have an impact on human health.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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