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Non-point source fecal contamination from aging wastewater infrastructure is a primary driver of antibiotic resistance in surface waters

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ABSTRACT

Antibiotic resistance is a global threat to human health. Many surface water resources are environmental hotspots of antibiotic resistant gene (ARG) transfer, with agricultural runoff and human waste highlighted as common sources of ARGs to aquatic systems. Here we quantified fecal marker genes and ARGs in 992 stream water samples collected seasonally during a 5-year period from 115 sites across the Upper Oconee watershed (Georgia, USA), an area characterized by gradients of agricultural and urban development. Widespread fecal contamination was found from humans (48% of samples), ruminants (55%), and poultry (19%), and 73% of samples tested positive for at least one of the six targeted ARGs (*ermB, tet*(B), $bla_{\text{CTX-M-1}}$, bla_{KPC}, bla_{SHV}, and *qnrS*). While ARGs were strongly correlated with human fecal markers, many highly contaminated samples were not associated with sewage outfalls, an expected source of fecal and ARG pollution. To determine sources of contamination, we synthesized ARG and fecal marker data with geospatial data on land use/land cover and wastewater infrastructure across the watershed. This novel analysis found strong correlations between ARGs and measures of sewer density, sewer length, and septic system age within sample watersheds, indicating non-point sources of fecal contamination from aging wastewater infrastructure can be critical disseminators of anthropogenic ARGs in the environment.

1. Introduction

The widespread use and over-use of antibiotics in human health and agriculture has made antimicrobial resistance (AMR) an ongoing global crisis (Antimicrobial Resistance Collaborators, 2022; O'Neill, 2016; World Health Organization, 2021). Resistance has been reported for nearly all known antibiotics (Ventola, 2015), and annual deaths from resistant infections are projected to reach 10 million by 2050 (O'Neill, 2016). To address AMR and develop mitigation strategies, it is critical to understand reservoirs and dissemination pathways of AMR. A growing body of research identifies the environment as both a recipient and source of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs). In particular, surface waters such as streams and rivers are an ideal setting for AMR dissemination, as they are not only impacted by anthropogenic activities but are also dynamic environments with a high potential for exchange of mobile genetic elements and

Abbreviations: ARB, antibiotic resistant bacteria; ARG, antibiotic resistance gene; AMR, antimicrobial resistance; PCA, principal component analysis; qPCR, quantitative PCR; UOWN, Upper Oconee Watershed Network; WWTP, wastewater treatment plant.

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ARGs, allowing for rapid spread of AMR throughout resident microbial populations (Amarasiri et al., 2020; Ben et al., 2019; Marti et al., 2014; Michael et al., 2013; Nappier et al., 2020; Nnadozie and Odume, 2019). The risk of resistant infections in humans is often related to the abundance and diversity of environmental ARGs, indicating links between the environment and the clinic (Bueno et al., 2017; Cho et al., 2020d; Finley et al., 2013; Nappier et al., 2020), though exact dissemination pathways largely remain unclear.

Fecal pollution from both animal and human waste are important sources of ARB and ARGs to surface waters. Determining sources of ARGs is particularly critical in mixed-use watersheds, which contain both urban and agricultural land and could thus receive ARGs from human and/or animal sources. Considerable amounts of antibiotics, ARGs, and ARB enter the environment from manure topdressing, runoff, or agricultural wastewater effluent (He et al., 2020; Nappier et al., 2020; Thanner et al., 2016), with wastewater from chicken and cattle farms often having ARG abundances comparable to hospital and municipal wastewater treatment plant (WWTP) effluent, and sometimes orders of magnitude greater (He et al., 2020). Human fecal contamination to surface waters includes both raw and treated waste. Raw sewage containing antibiotics, ARGs, and ARB (often including pathogens) can enter surface waters through aging or inadequate wastewater infrastructure (Eramo et al., 2017; Gallert et al., 2005; Ikhimiukor et al., 2022; McLellan and Roguet, 2019; Nappier et al., 2020), typically considered non-point source contamination due to its distributed nature. Furthermore, treated sewage typically contains detectable ARB, ARGs, and human fecal indicators (Amos et al., 2018; Wéry et al., 2010; Wu et al., 2020), leading to release of ARB and ARGs into the environment via effluent discharge, a common point source of these contaminants worldwide (Berglund et al., 2015; Buelow et al., 2020; Cacace et al., 2019; Karkman et al., 2018; Osińska et al., 2020). In some cases sewage effluent even becomes enriched in ARGs through the treatment process (Amos et al., 2018; Bengtsson-Palme et al., 2019; Di Cesare et al., 2016; Osińska et al., 2019).

While numerous studies have reported on either agricultural waste or sewage effluent as an ARG source to surface waters, few studies have assessed their relative impact or quantified the relative importance of point source and non-point source fecal contamination to ARG dissemination. We collected a multi-year seasonal time series of fecal marker and ARG abundance at high spatial resolution throughout the Upper Oconee watershed (northeast Georgia, USA; Figs. S1, S2) (Fisher et al., 2000), combined with data on geospatial land use/land change and wastewater infrastructure in the watershed. The Oconee River is the primary source of drinking water for Athens-Clarke County and is widely used for recreation. Its mixed-use watershed is analogous to other similarly sized watersheds around the world, containing forested, agricultural, industrial, and residential areas subject to increasing urbanization. Many tributaries in the watershed begin as relatively undeveloped headwater streams, then flow through areas impacted by agricultural runoff from farmland and poultry processing before entering suburban regions and a central urban area (Athens), where they receive effluent from multiple WWTPs (Figs. S1, S2). Previous studies have documented pathogenic and potential epidemic strains of ARB in the Upper Oconee watershed (Cho et al., 2020a; 2020b; 2022; 2018; 2020c; 2019; Meinersmann et al., 2008; 2013), suggesting its surface waters are a significant reservoir and source of AMR. However, the origins of anthropogenic ARGs in these waters remain unknown.

To examine relationships between fecal contamination and ARGs in surface waters across this watershed, quantitative PCR (qPCR) for fecal source tracking genes and ARGs was conducted on samples from 5 years of quarterly water quality monitoring (2013-2018; n=992), as well as on samples of influent and treated effluent from 3 WWTPs (n=17; Fig. S2). To pinpoint specific sources of fecal and ARG contamination, we used correlation analyses and ordinations to evaluate spatial relationships between fecal indicators/ARGs and geospatial estimates of wastewater infrastructure and land use/land cover across the watershed. This unique synthesis of data on ARG abundance, fecal source tracking, land use/land cover, and wastewater infrastructure allows for unprecedented determination of surface water fecal and ARG contamination sources throughout a mixed-use watershed, highlighting non-point source contamination from sewers and septic systems as important sources of fecal and ARG contamination to the environment.

2. Materials and methods

2.1. Sample collection

The 115 sites sampled throughout the Upper Oconee Watershed (Fig. S3) were established by the Upper Oconee Watershed Network (UOWN), a local water quality monitoring organization. Quarterly samples were collected in collaboration with trained UOWN citizen scientists from Spring 2013 (April 20th) to Summer 2018 (July 8th), resulting in collections across 22 total seasons. The number of sites collected each season (range: 16-90, mean: 45, standard deviation: 22) was dependent on the number of volunteers. Filters for planktonic DNA were collected as previously described (Hassell et al., 2018). Briefly, water samples were stored on ice prior to filtration within 5 hours of collection. Water was filtered by peristaltic pumping through a 5.0 μm Durapore SVPP pre-filter (Millipore, Billerica, MA, USA), then a 0.22 μm Sterivex filter (Millipore; mean±standard deviation, 852±460 mL filtered). For WWTP influent samples, multiple pre-filters and 0.22 μm filters were used per sample to maximize total volume filtered due to high particulate matter loads. All filters were stored dry at -80°C prior to DNA extraction.

2.2. DNA extraction and gene abundances

DNA extraction from the 0.22 μm filters was completed as previously outlined (Hassell et al., 2018). Briefly, on-filter lysis proceeded using lysozyme, then a proteinase K + SDS digestion. Resulting lysate was extracted using phenol:chloroform:isoamyl alcohol (25:24:1; pH 8.0), precipitated with isopropanol, and resuspended in 400 μl of elution buffer (Omega BioTek, Norcross, GA, USA) and purified with the E.Z.N.A. Water DNA Kit (Omega Biotek, May 2013 version), starting with the cHTR buffer treatment step. All samples were eluted in 50 μl of elution buffer. DNA concentrations and quality were measured using a Qubit 2.0 fluorometer (HS assay; Thermo Fisher, Waltham, MA, USA) and a NanoDrop Lite spectrophotomer (Thermo Fisher), respectively.

Given the large number of samples, it was not feasible to quantify a broad diversity of ARGs via shotgun metagenomics or high-throughput qPCR arrays. An initial set of 15 DNA samples (13 from streams, 1 WWTP influent, and 1 WWTP effluent) were used with Antibiotic Resistance Genes Microbial DNA qPCR Arrays (QIAGEN, Germantown, MD, USA) targeting 88 ARGs or ARB. Of these, 6 ARGs that were relatively abundant across initial samples (Table S1), represented resistance to a breadth of antibiotic classes, and/or were documented in strains isolated from the same watershed (Cho et al., 2020c; 2020a; 2022; 2019; 2018), were selected for targeted analyses: *ermB* (macrolide-lincosamide-streptogramin B resistance), *tet*(B) (tetracycline resistance), $bla_{\text{CTX-M-1}}$ (ß-lactam resistance), bla_{KPC} (ß-lactam resistance), bla_{SHV} (ß-lactam resistance), and *qnrS* (quinolone resistance).

Extracted DNA was used with qPCR assays for total bacterial 16S rRNA genes, fecal source indicators (human, ruminant, and poultry), and the 6 selected ARGs (Table S2). Standards for bacterial 16S and source-tracking markers consisted of a linearized plasmid. ARG assays were run in duplex using FAM and VIC probes (Table S2), using synthetic gBlock gene fragment standards (Integrated DNA Technologies, Coralville, IA). All reactions were set up in triplicate with the TaqMan™ Environmental Master Mix 2.0 (Thermo Fisher) and were run on a QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher). Standard curves (consisting of a dilution series of linearized plasmids or gBlock fragments) were run on every plate. An internal TaqMan™ Exogenous

Internal Positive Control (Thermo Fisher) was used to monitor inter-assay variability.

A gene was considered "detectable" if at least two technical replicates had C_T values greater than those of the lowest standard (see Table S2). If standard deviation between technical triplicates was >0.55 C_T (the lowest value obtained in standard curves), an outlier was removed; the sample was rerun if the spread remained >0.55 C_T. Gene abundances were reported as gene copies mL^{-1} site water. Total ARG abundance represents the sum of the abundance of all 6 individual ARGs. For some samples, DNA was depleted prior to running all qPCR assays; assays not run for this reason were marked as "NA" (whereas those run but below the detection limit were recorded as "0").

2.3. Geospatial analyses

Geospatial data were calculated using ArcMap 10.x (Esri, Redlands, CA). Watersheds for each sampling location were delineated using the hydrology toolbox. The National Elevation Dataset's 10m digital elevation model was used to calculate geomorphic characteristics of each sample point's watershed (e.g., slope, total stream length, ruggedness) (McDonald et al., 2018). Land use/land cover for the watershed draining to each sample point were extracted from the 2016 National Land Cover Dataset (Homer et al., 2020). High-quality geospatial data on septic and wastewater infrastructure in Athens-Clarke County was accessed through a partnership with the Athens-Clarke County Public Utilities department and were extracted from the Athens-Clarke County public works datasets as previously described (Capps et al., 2020). Site-linked sewer and septic data are withheld due to potential privacy concerns. Road lengths/densities and relations to sample sites were calculated using the US Census TIGER lines streets dataset.

2.4. Statistical analyses

Statistical analyses were conducted in R (R Core Team, 2019), with most plots made using ggplot2 (Wickham, 2016). Histograms and violin plots were used to assess the co-occurrence of ARGs and source tracking genes. Samples were split into groups based on the detectability of each source tracking gene; between these groups, histograms compared the number of detectable ARGs and violin plots compared the aggregate abundance of ARGs. Differences in the sum of ARGs between samples with and without detectable source tracking genes were determined using the Mann-Whitney *U* test due to non-normal distributions of some data. Correlations (Spearman's ρ) between source tracking gene abundance and the sum of ARG abundances were calculated using the Hmisc R package (Harrell, 2019). For all correlation analyses, Benjamini-Hochberg corrected *p*-values are reported to correct for multiple comparisons.

Site-averaged data were compared using scatterplots, regressions, and correlation analyses. The qPCR data (genes per mL^{-1} water) were $\log_{10}(x+1)$ transformed, then averaged by site. Model II regressions of site averaged data were calculated using the lmodel2 R package (Legendre, 2018) as previously described (Damashek et al., 2019). Pairwise correlations (Spearman's ρ) between site-averaged qPCR, land use/land cover, road, sewer, and septic variables were calculated using Hmisc. Correlations with corrected $p<0.05$ were considered significant. Hierarchical clustering was conducted using the *hclust()* R command with complete linkage. Heatmaps were drawn using ggcorrplot (Kassambara, 2019), with insignificant correlations crossed out. An initial analysis including all environmental variables identified clusters of geomorphic variables highly correlated with one another, which largely differentiated small versus large basins (Fig. S4). Guided by these groupings, geomorphic variables other than ruggedness, melton ruggedness ratio, and mean total stream length were removed and correlations analyses were rerun. Similarly, given their high level of correlation, land use/-land change variables estimating developed land (open, low, medium, or high) were combined into one aggregate "Developed" estimate, variables estimating forest cover (deciduous, evergreen, or mixed) were combined into one "Forest" estimate, and grassland and pasture variables were combined into one "Grassland" estimate.

To determine whether watershed-scale correlations primarily reflected extreme values at specific sites, we reran correlation analyses with samples from Brooklyn Creek excluded. Brooklyn Creek has been considered a critically impaired creek with consistently high *E. coli* counts for over a decade, leading to its listing as a 303(d) threatened waterway by the US EPA. This stream could also be impacted by compromised sewage infrastructure from the two main hospitals in the region. Furthermore, Brooklyn Creek samples consistently had the highest abundance of ARGs and human fecal markers in our data.

A principal component analysis (PCA) was calculated with the *PCA()* function from FactoMineR (Lê et al., 2008), using $\log_{10}(x+1)$ transformed gene abundances as input. PCA results were visualized using the *fviz_pca_biplot()* function from factoextra (Kassambara and Mundt, 2017) with scaling 1.

3. Results

Most sites across the watershed were sampled repeatedly (Fig. S3), and approximately half of the 992 resulting samples had detectable human (48%) or ruminant (55%) fecal genes while only 19% had detectable poultry genes (Table S3). Of the ARGs tested, *ermB* was detected most frequently (73.8% of samples), $bla_{CTX\text{-}M\text{-}1}$ was rarest (2.6%), and the other four genes were found in 24.9-49.9% of samples (Table S3). When the data were split by the presence of each source tracking gene, ARGs were always enriched in samples where the source tracking gene was detected (Mann-Whitney *U* test, $p<0.001$; Fig. 1A). Furthermore, human and ruminant fecal contamination were associated with a higher likelihood of detecting one or more ARGs, and all samples where all 6 targeted ARGs were detected ($n=33$) also had detectable human fecal contamination (Fig. 1B).

ARGs and human fecal genes were highly correlated in both surface water (Spearman's $\rho=0.632$, $p<0.001$) and WWTP influent/effluent (Spearman's $\rho=0.931$, $p<0.001$; Fig. 2, Table S4). Correlations between human fecal genes and ARGs for samples collected during each sampling year were all highly significant (all Spearman's $\rho\geq0.466$, $p<0.001$; Fig. S5). ARGs were less strongly correlated with ruminant and poultry genes (Spearman's $\rho=0.308$ and 0.372, respectively; Fig. 2, Table S4). While still detectable, abundance of ARGs and human fecal genes in WWTP effluent was lower than untreated sewage influent (Fig. 2). Though samples collected downstream from WWTP outfalls had somewhat high human fecal and ARG abundance, the greatest abundances for both ($\sim10^7$ genes mL^{-1}) were in samples collected from locations upstream of WWTP outfalls (Fig. 2). When gene abundance was averaged across all time points at each site, human fecal genes and ARGs remained linearly correlated ($F_{1,110}=214$, $p<0.001$, $r^2=0.658$; Fig. 3). Similar to single samples, the highest site-averaged ARG and human fecal gene abundance ($\sim10^3$ genes mL^{-1}) were at sites that were *not* downstream of WWTP outfalls.

To further analyze the relationships between source tracking genes and ARGs, we conducted a PCA to group samples by gene abundance. PC1 explained 73.0% of variation and primarily consisted of effects from aggregate ARG, human fecal, and most individual ARG abundances, whereas PC2 (11.3% of variation explained) primarily represented ruminant fecal gene abundance (Fig. 4). Therefore, PC1 appears to largely reflect human fecal contamination and associated genes, whereas PC2 reflects the influence of livestock production, particularly cattle. Loadings of all ARGs were positively correlated with PC1, suggesting all ARGs generally increased as human fecal markers increased, while their loadings on PC2 were both positive and negative, with *tet*(B) having the highest positive correlation to PC2. Notably, the PCA did not show distinct clusters between sites located upstream or downstream of WWTP outfalls.

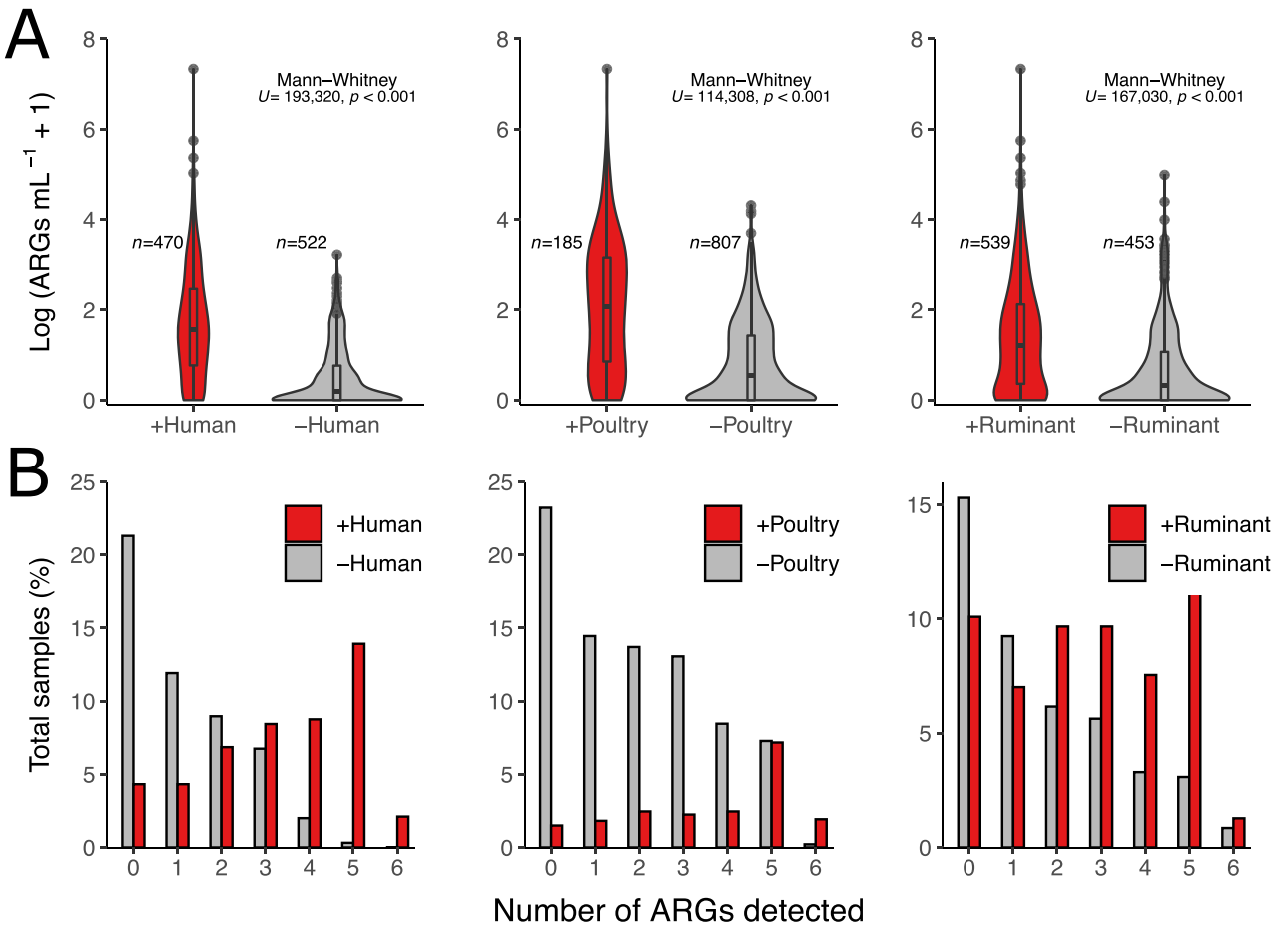

Fig. 1. Distributions of detected ARGs, with samples split by detection of source tracking genes. Red indicates detection (+) while gray indicates no detection (–) of each source tracking gene. **A)** Violin plots of the sum of ARG abundances. Overlain boxplots show the median, interquartile range, and outliers. **B)** Histograms of the fraction of samples that were positive or negative for each source tracking marker from which 0-6 ARGs were detected.

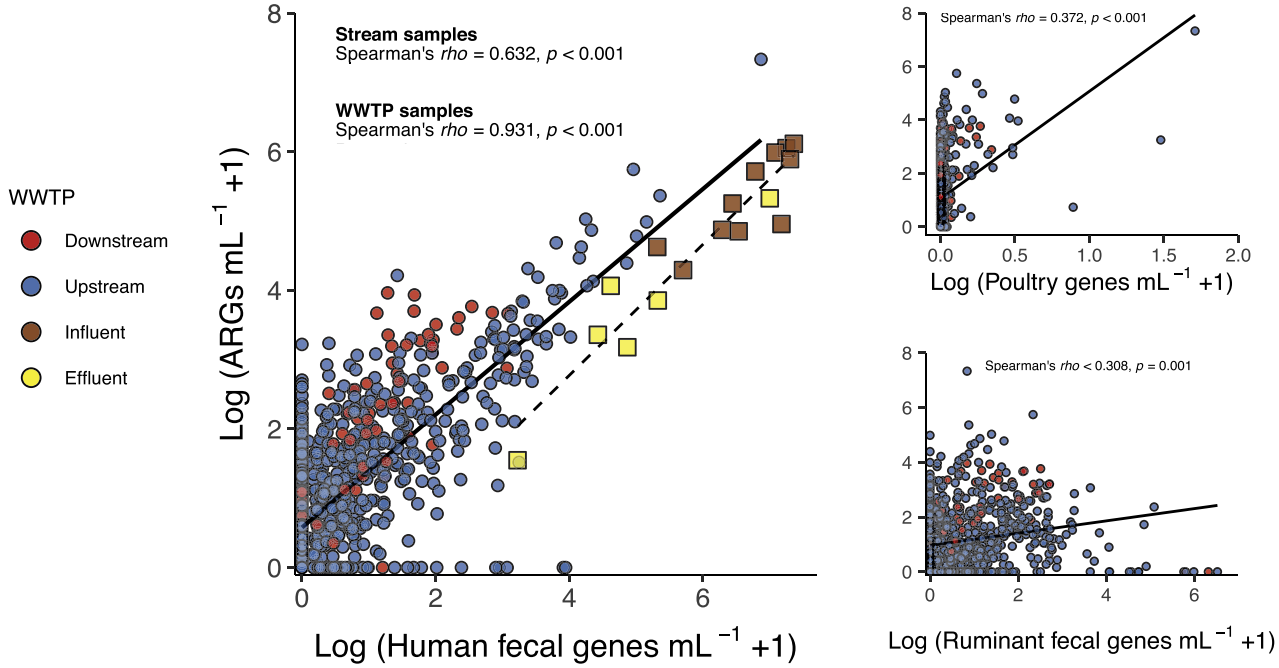

Fig. 2. Correlation of ARGs with human, poultry and ruminant fecal markers. Circles are stream samples (n=992), with color denoting location downstream (red) or upstream (blue) of a WWTP outfall (human plot: n=944 stream samples, poultry: n=934, ruminant: n=941). In the human plot, squares show WWTP influent (brown; n=11) and effluent (yellow; n=6). Solid black lines show linear fits, with the dashed line in the human plot showing a linear fit of WWTP samples only.

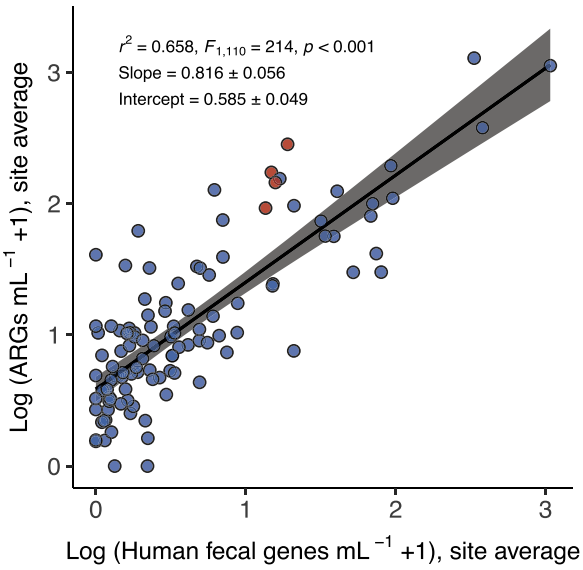

Fig. 3. Site averaged ARG and human fecal gene abundance. Red points (n=4 of 112) are downstream from WWTP outfalls. The black line shows a linear fit and gray shading shows a 95% confidence interval.

We tested the impact of removal of Brooklyn Creek samples from the dataset to ensure these relationships were not the result of a few outlying samples. The Brooklyn Creek watershed contains two large hospitals (Fig. S6) and was the most highly contaminated single stream in terms of human fecal genes (Fig. S7), which were highly correlated with all measured ARGs in Brooklyn Creek samples (Fig. S6). However, when Brooklyn Creek samples were removed from the dataset and correlations recalculated, the strong correlation between ARGs and human fecal genes remained (Fig. S8).

To evaluate possible sources of contamination, we examined correlations between human fecal genes or ARGs and measures of land use/land cover and urban infrastructure in the watershed of each sampling location. All variables significantly correlated to ARGs were also significantly correlated with human fecal genes (Figs. 5, S9, S10). The strongest correlations were between human fecal genes or ARGs and markers of urban infrastructure: in particular, site-averaged human fecal genes, total ARGs, and many individual ARGs were correlated to estimates of road and sewer length, road and sewer density, and road crossings within sample watersheds (Figs. 5, 6, S9, S10). Of the measured ARGs, bla_{SHV} and bla_{KPC} had strong correlations with these variables (nearly the same as human fecal genes; Fig. S9, S10). Additionally, there were significant correlations of average septic system age within sample watersheds with site-averaged human fecal genes (Spearman's ρ=0.376, p=0.001) and ARGs (Spearman's ρ=0.349, p=0.003; Figs. 5, 6, S10). When Brooklyn Creek samples were removed, both correlations with average septic age were no longer significant (Fig. S11).

4. Discussion

4.1. Human fecal contamination as a source of ARGs

Aquatic ecosystems can harbor human and animal pathogens, with fecal pollution being a major contaminant source in rivers worldwide (Amarasiri et al., 2020; Bueno et al., 2017; Marti et al., 2014). Furthermore, freshwater systems are considered active "bacterial genetic reactors" ideal for horizontal transfer of ARGs between autochthonous microorganisms and pathogens (Baquero et al., 2008; Marti et al., 2014). Though multiple sources of fecal pollution exist at the watershed level, most can be generalized into two categories: (1) agricultural, primarily runoff from livestock waste (He et al., 2020), and (2) human waste from sewage systems, septic systems, and WWTPs (Gonzalez et al., 2020; Rizzo et al., 2013; Sowah et al., 2017). Pinpointing sources of fecal pollution and ARGs in mixed-use watersheds (containing both urban and agricultural land) is a critical global challenge to guide natural resource policy and stem the environmental spread of AMR.

A striking observation from the Upper Oconee data is that human fecal contamination (found in nearly half of the samples) was highly correlated with ARG abundance throughout the watershed (Fig. 2). In contrast, correlations between ARGs and either ruminant or poultry fecal markers were much weaker, indicating human fecal contamination as the primary source of ARG contamination to surface waters throughout the watershed. Though agricultural sources likely contribute some ARGs, their impacts appear minimal relative to human waste. This observation is supported by a recent meta-analysis of shotgun metagenomes, which also found an association between human fecal and ARG contamination in environments with high anthropogenic impacts (Karkman et al., 2019), as well as similar relationships found in other aquatic ecosystems (Sala-Comorera et al., 2021; Stachler et al., 2019; Thongsamer et al., 2021; Young et al., 2013). Human fecal contamination of surface waters is widespread across the globe and is often linked to gastrointestinal disease (Boehm et al., 2015; Chen et al., 2019; Eramo et al., 2017; Jennings et al., 2020; Sauer et al., 2011). The strong association between human fecal contamination and AMR in our data suggests this contamination might represent an underappreciated public health risk, and adds urgency to calls for improved risk models for environmental AMR exposure (e.g., Leonard et al., 2022; Nappier et al., 2020) and more precise quantification of the epidemiology of infections acquired from environmental AMR (e.g., Finley et al., 2013; Stanton et al., 2022).

Another important trend to notice is that the abundances of all 6 selected ARGs were highly correlated with one another and all showed similar relationships to the three fecal markers and environmental factors (Figs. 4, 5, S9, S10). This suggests common sources and/or selective pressures, despite the fact that we selected ARGs targeting three different antibiotic classes (ß-lactams, tetracyclines, and quinolones). In selecting markers, we focused primarily on genes associated with human pathogenesis. For example, 4 of the 6 targeted ARGs are designated as "Type I" threats (Zhang et al., 2021), being associated with human pathogenesis, mobile genetic elements, and environments with high human influence. Of the exceptions, *tet*(B) is also human-associated but not typically mobile. The other exception, $bla_{CTX\text{-}M\text{-}1}$, was not included in that framework, but we note that other $bla_{CTX\text{-}M}$ groups are listed as Type I, and $bla_{CTX\text{-}M\text{-}1}$ has become a widespread ESBL (e.g., Amos et al., 2014; D'Andrea et al., 2013; Zurfluh et al., 2014) and is transferred via plasmids, so we consider $bla_{CTX\text{-}M\text{-}1}$ as "high risk" as well. While this selection process may in part explain the tight correlation with human fecal contamination rather than agricultural sources, this relationship is nonetheless important to note, given the public health importance of these ARGs.

Previous work has demonstrated that ARG diversity and abundance can differ between surface waters and associated flocs or suspended sediments, with surface sediments and flocs originating from WWTP effluent often hosting enriched and distinct ARGs compared to bulk water (Brown et al., 2019; Calero-Cáceres et al., 2017; Luo et al., 2010). Since this study targeted the "free-living" microbial fraction (<5.0 μm), we are unable to speculate on differences between free-living and particle-associated ARGs in this system. Therefore, it remains unknown whether differences exist between size fractions, or whether stream sediments represent an enriched reservoir of ARGs throughout this watershed.

4.2. Relative contributions of point and non-point sources of human fecal contamination

To effectively mitigate ARG pollution, sources of human fecal contamination to surface waters must be identified. As is typical for

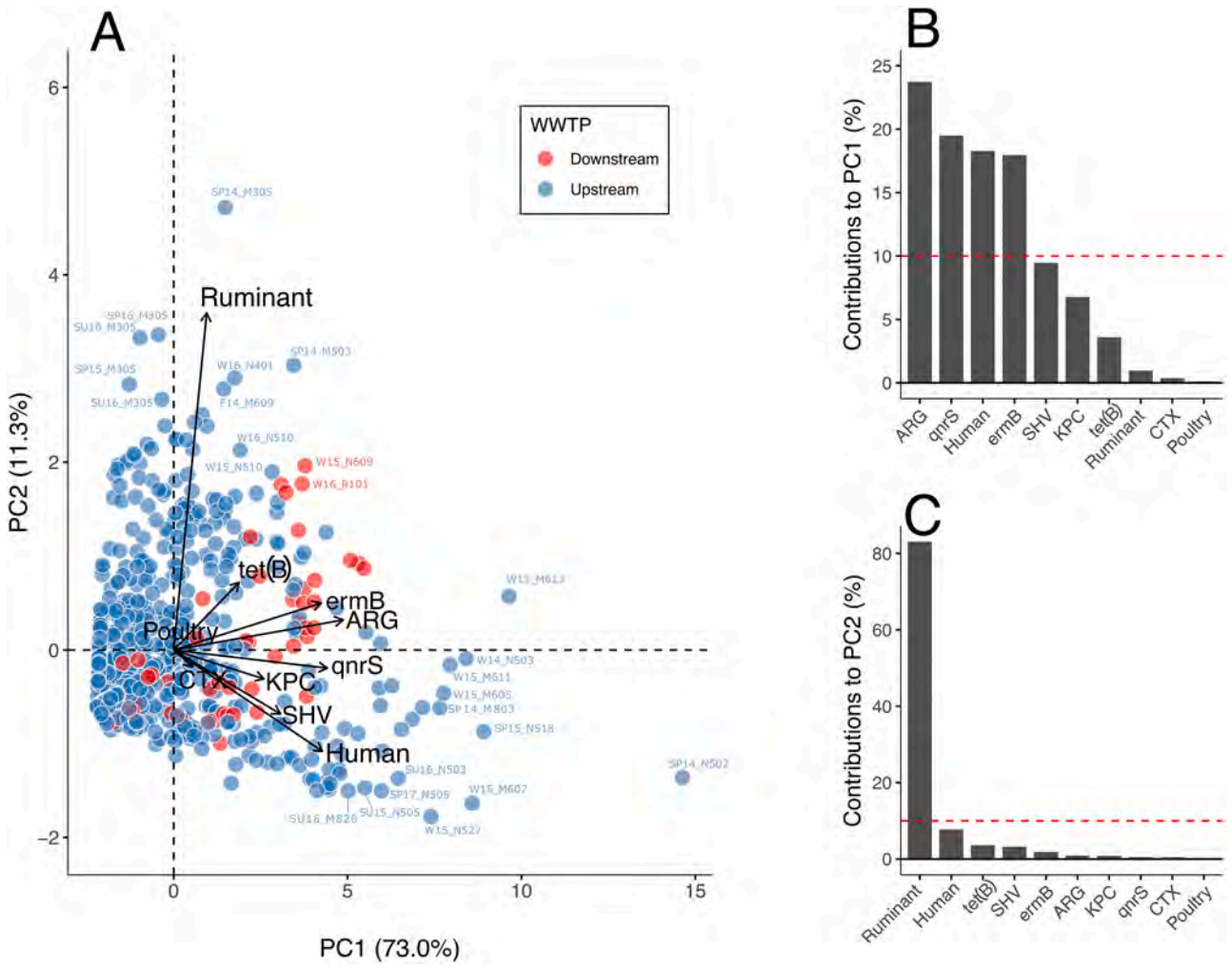

Fig. 4. PCA of gene abundances. **A)** Points represent samples (n=692). Arrows show the direction of increasing gene abundance. Samples at the periphery are labeled. **B, C)** Contributions of the abundance of each gene to PC1 (B) and PC2 (C). The dashed red line shows the expected contribution of each variable if all contributed to the PC randomly (10%). Abbreviations: "CTX"= $bla_{\text{CTX-M-1}}$, "KPC"=bla_{KPC}, "SHV"=bla_{SHV}. "Human," "Ruminant," and "Poultry" represent the respective source tracking markers.

mixed-use watersheds, there are numerous possible sources of human fecal contamination to Upper Oconee waters, including WWTP effluent, leaking sewer pipes, and failing septic systems. We initially hypothesized that WWTP effluent would be the primary source of ARGs, as WWTPs are generally considered hotspots for AMR (Guo et al., 2017; Marti et al., 2014; Rizzo et al., 2013) and the release of WWTP effluent into receiving waters is often an important point source of ARB and ARG release into the environment (Nappier et al., 2020; Rizzo et al., 2013). While treatment at WWTPs in our watershed reduced molecular markers for human fecal markers and ARGs, both remained present at varying levels in treated effluent (Fig. 2), suggesting a potential source to nearby waters. However, human fecal and ARG contamination were not restricted to WWTP outfalls, and, surprisingly, many sites with the highest contamination levels were *not* downstream of WWTP outfalls and included headwater streams (Figs. 2, 3). Furthermore, the PCA showed no distinct clustering of downstream compared to upstream sites (Fig. 4). This suggests that WWTP effluent, a common point source of fecal and ARG contamination globally (e.g., Brown et al., 2019; Munir et al., 2011; Murphy et al., 2021), is not a primary driver of AMR (or human fecal contamination) in much of this watershed.

Where, then, is the fecal and ARG contamination in the Upper Oconee coming from? Using a unique combination of landscape-level qPCR (collected over time from numerous sites across the watershed), land use/land cover, and sewer/septic density data, we found this pollution was most closely associated with sewage infrastructure: both human fecal genes and ARGs were highly correlated to the sewer density, the density of sewer crossings, and road density within sample watersheds (Fig. 5). The correlations of fecal genes with sewer density and sewer crossings indicate failures in the sewer transport system may be a major source of fecal and ARG contamination to the environment. The association of road density to human fecal and ARG detection (Figs. 5, 6) can be explained by modern sewerage systems constructed under or in close proximity to roadways, as well as links between road density and general population/infrastructure density. More specifically, there were strong positive correlations between numerous measures of roads and sewers associated with a site (road length, road density, sewer density, sewer length, and sewer crossings) and the site-averaged abundance of the ß-lactam resistance genes bla_{KPC} and bla_{SHV}, suggesting sewers are a significant source of these ARGs. Furthermore, all measured ARGs except the tetracycline-resistance gene *tet*(B) were positively correlated with at least one variable related to sewer infrastructure (Fig. 5), suggesting sewerage as a widespread source of ARG contamination to Upper Oconee surface waters. This complements the growing literature documenting the association of ARB and ARGs with sewers (Auguet et al., 2017; Fahrenfeld and Bisceglia, 2016; Medina et al., 2021), suggesting the high levels of AMR present in sewer systems can increase AMR in nearby environments. It is unclear why *tet*(B) alone is uncorrelated from measures of sewer infrastructure. Tetracycline is

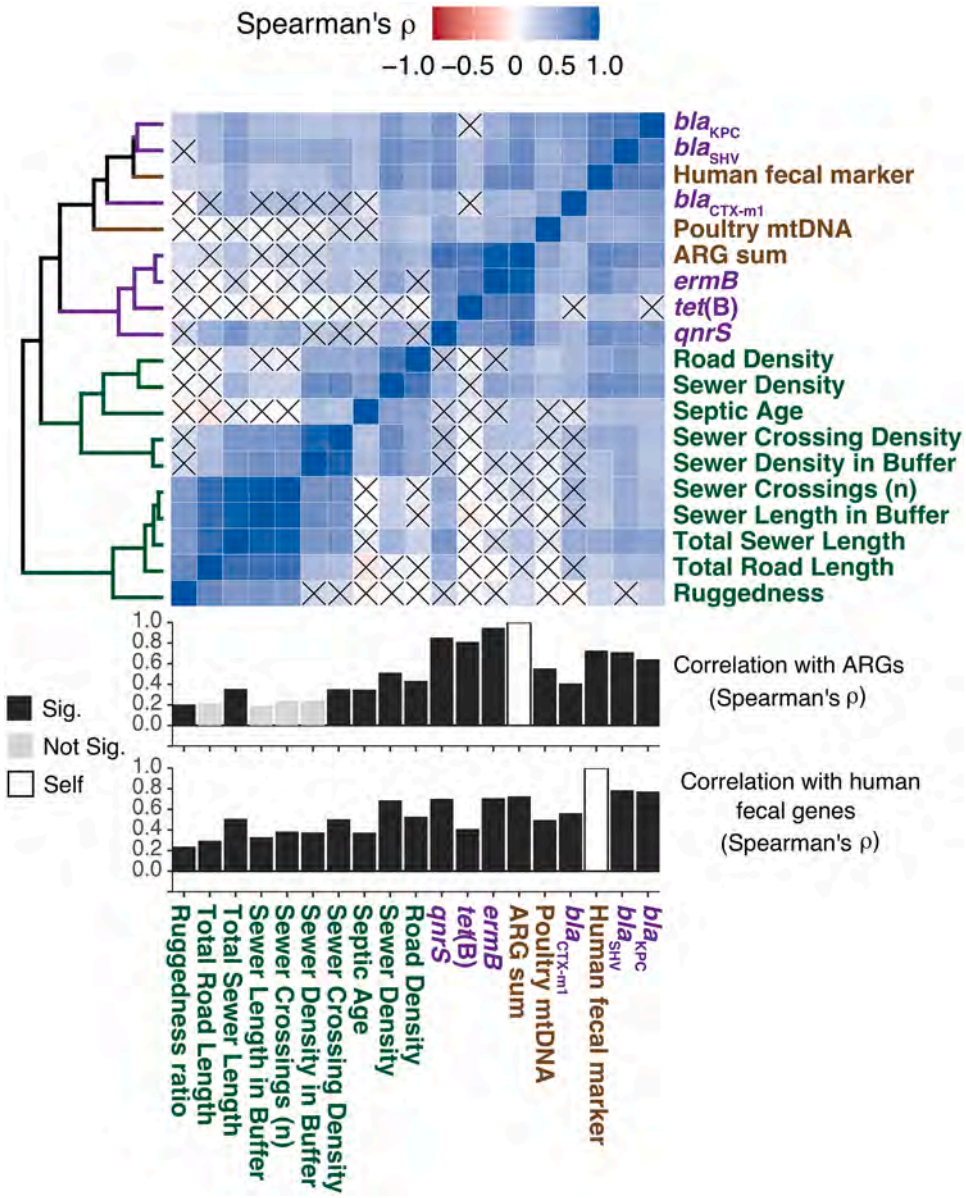

Fig. 5. Correlation of fecal markers, antibiotic resistance markers, and geospatial attributes. Only variables significantly correlated with either ARGs or human fecal genes are included. Top shows correlations (crossed out if $p>0.05$). The dendrogram indicates hierarchical clustering. Bottom: correlations of variables with ARGs and with human fecal genes. All qPCR data is based on site-average log10 copies mL^{-1}, landscape variables are in km (length measures), km × km^{-2} (density measures), years (average septic system age), or are unitless (ruggedness ratio, sewer crossings). Sewer density and sewer length in buffer are calculated within a 20 m buffer.

highly used in livestock production, and *tet* genes are often associated with agricultural sources (e.g., Luo et al., 2011; Singer et al., 2016; He et al., 2020). This is reflected in our PCA analyses, where *tet*(B) had the strongest positive contribution of any ARGs to PC2, an axis largely representative of agricultural influence (Fig. 4). This may suggest difference sources of *tet*(B) sources than the other targeted ARGs throughout the watershed, potentially reflecting a greater influence of agriculture and livestock production on *tet*(B) in this environment.

Decentralized wastewater systems (such as septic tanks) can also have a large impact on surface water fecal contamination, especially seasonally (Sowah et al., 2014, 2017). These systems are a critical part of wastewater treatment infrastructure throughout the globe, including in the Upper Oconee watershed, where ~42% of developed parcels use decentralized wastewater treatment systems (Capps et al., 2020). We leveraged the unique data on septic density and septic age in the Upper Oconee watershed (Capps et al., 2020) to investigate the relationship between septic systems with human fecal and ARG contamination. While neither human fecal genes nor ARGs were significantly correlated with overall septic density, site averages of both were significantly correlated with the average septic age within sampling watersheds (Figs. 5, 6), suggesting aging septic systems contribute to surface water fecal and ARG contamination across the Upper Oconee watershed. Like sewer variables, septic age was significantly correlated with bla_{KPC} and bla_{SHV} abundance. Most (70%) septic systems within the Upper Oconee watershed are potential environmental hazards due to their age (>25 years, with 28% of systems at >45 years old) (Capps et al., 2020), with our data indicating these aging septic systems are a source of fecal and ARG contamination specifically. Studies of drain field effluent acceptance rates suggest septic systems can remain hydraulically operational for approximately 11 to >30 years (Siegrist et al., 2000), but half of septic systems 19 to 27 years old are expected to show evidence of failure (Clayton, 1974; Winneberger, 1975). Furthermore, these failures may be clustered in space and time since septic systems are typically installed in clusters.

Brooklyn Creek (see Fig. S6) is a representative example of a headwater stream in this watershed that has no WWTP effluent impact yet had high human fecal and ARG contamination (Fig. S7). Numerous samples collected from Brooklyn Creek even had comparable or greater numbers of human fecal markers and ARGs than untreated sewage from WWTP influent, suggesting failing wastewater infrastructure is a

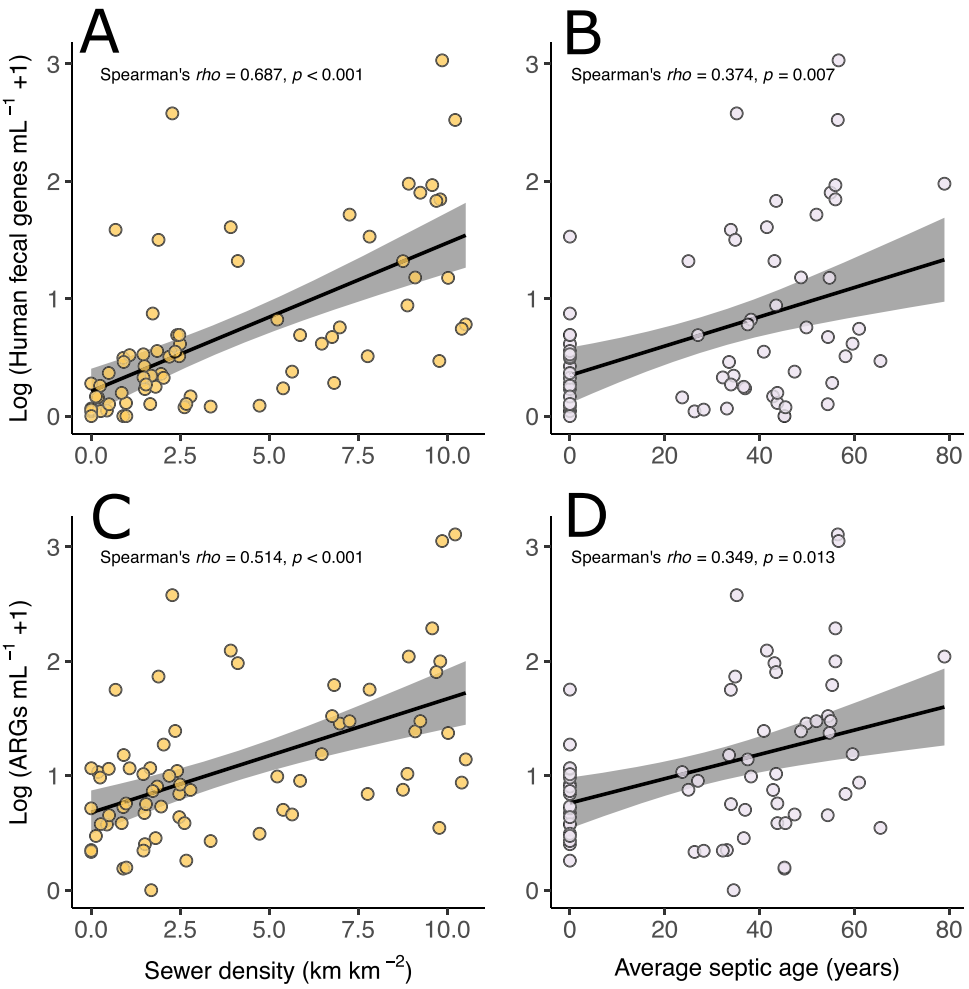

Fig. 6. Human fecal markers and ARGs compared to sewer density and septic tank age. Scatterplots of **A**) sewer density and **B**) average septic age versus site average human fecal genes, and **C, D**) versus the site average sum of ARGs (n=112). qPCR measurements from each year were log10(x+1) transformed, then averaged by site. The black line shows a linear fit, with gray shading representing a 95% confidence interval.

significant issue along this particular waterway. When Brooklyn Creek samples were removed from analyses, correlations between average septic age and ARGs or human fecal genes were weaker and no longer statistically significant (Fig. S11). However, human fecal markers and ARGs, as well as sewer density and both ARGs and human fecal genes, remained highly correlated (Figs. S8, S11). This indicates septic systems within the Brooklyn Creek sub-watershed were an important driver of these patterns across the Upper Oconee watershed.

4.3. Agricultural sources of ARGs in the watershed

Prior work has demonstrated that manure can be an important propagation pathway of AMR in the environment, including studies demonstrating livestock waste as a potential source for ARGs and ARBs to a variety of surface water ecosystems around the globe (Chee-Sanford et al., 2009; Hagedorn et al., 1999; He et al., 2020; Keenum et al., 2021; Thanner et al., 2016). Given the preponderance of agricultural production areas throughout the Upper Oconee watershed, including cattle pastures and a high density of poultry production facilities, we sought to determine the relationship between ARG abundance and ruminant or poultry waste throughout this watershed. However, ruminant fecal and poultry genes were only weakly correlated with ARGs at the watershed scale (Fig. 2), indicating a relatively minor role for agricultural waste as a source of ARGs throughout the Upper Oconee. The poultry mitochondrial DNA marker gene used in this study is highly specific to chicken as compared to other avian primers (Boehm et al., 2013; Ryu et al., 2014; Schill and Mathes, 2008). However, since it targets mitochondrial DNA, it is not specific to poultry feces and could potentially be associated with non-fecal DNA sources such as industrial poultry processing or residential/restaurant drain disposal of meat associated with food preparation or consumption. This is supported by weak but significant correlations of the poultry marker with the human fecal marker and with sewer density (Fig. S4), indicating a correlation between human population density and poultry marker abundance in surface waters. Yet despite the abundance of poultry production in the area, this marker was detected in only 19% of the samples and was not strongly correlated with ARGs, suggesting industrial poultry processing was not a significant source of surface water ARGs within the watershed.

4.4. Consequences for AMR mitigation

Quantifying antibiotic resistance transmission risk from the environment to humans is critical for stemming clinical risk associated with AMR (Ikhimiukor et al., 2022; Manaia, 2017). ARG presence and diversity in clinical settings is often similar to that of nearby environments and/or WWTP effluent, suggesting rapid transmission routes from the environment to medical settings (Ekwanzala et al., 2018; Pärnänen et al., 2019). Furthermore, analysis of historical AMR data found spikes in environmental detection of many ARGs 1-2 years prior to their detection in clinical settings, suggesting high environmental ARG abundance may portend increased clinical risk (Hua et al., 2020). Determining the factors driving ARG prevalence in the environment is

therefore critical for understanding and mitigating AMR exposure to human populations.

The risk of AMR transmission from the environment to humans must be assessed on whether there are ARBs capable of colonizing the human body, rather than ARGs alone (Manaia, 2017). Although this study quantified ARGs irrespective of their genomic context, we note that a series of studies carried out in the same watershed using concurrently collected water samples for culture based characterization of isolates showed widespread presence of antibiotic resistant *Salmonella, Escherichia coli,* and *Enterococcus* (Cho et al., 2020c, 2020a, 2022, 2019, 2018). The *E. coli* isolates from the Upper Oconee included the epidemic strain ST131, known to pose serious global health risks (Johnson et al., 2010), and included an isolate from the Brooklyn Creek watershed exhibiting the dangerous extended-spectrum ß-lactamase phenotype (Cho et al., 2020b). This body of research indicates the surface water ARGs documented here may indeed be associated with ARB, including some that pose a significant public health risk.

Our findings highlight human fecal and ARG contamination of surface waters as a direct hazard of aging or failing infrastructure such as sewage and septic systems. This problem is not unique to this watershed, as aging and failing wastewater infrastructure is a chronic problem across the United States (ASCE, 2021; Selvakumar and Tafuri, 2012), and similar infrastructure failures are emerging as a major pathway of surface water contamination for a variety of pollutants (Fork et al., 2021; Gallert and Winter, 2005; Gonzalez et al., 2020). The U.S. is in a nationwide infrastructure crisis, with wastewater infrastructure nationally earning an extremely low D+ on the American Society of Civil Engineers infrastructure report (ASCE, 2021). A critical corollary of our findings is that the modernization of WWTPs alone will not be sufficient to eliminate surface water fecal contamination and ARG contamination. To this end, there is an urgent need for capital investment in sewage conveyance and septic infrastructure to protect public health and the environment.

Many existing AMR action plans lack any significant focus on the environment, potentially due to the difficulty of satisfactorily answering fundamental questions about the sources and dynamics of environmental AMR (Singer et al., 2016). Most studies assessing sources of ARGs to surface waters have focused on WWTPs or agricultural inputs (Amarasiri et al., 2020; Ben et al., 2019; Bueno et al., 2017; He et al., 2020; Marti et al., 2014; Michael et al., 2013; Nappier et al., 2020; Nnadozie and Odume, 2019), likely leading to sampling bias toward locations near these point sources. By studying an entire watershed over time instead of primarily focusing on waters directly downstream of known WWTP or agricultural inputs, we demonstrate human fecal pollution from non-point sources such as aging sewage and septic infrastructure plays an important role in AMR dissemination to surface waters. These systems should therefore be considered and potentially prioritized in public health efforts addressing the rise in AMR, and programs studying ARG sources to the environment should include a diversity of sites across the ecosystem to ensure against missing this potentially important source of contamination.

5. Conclusion

Fecal contamination from human and agricultural waste is a major contributor of antibiotic resistant bacteria and antibiotic resistant genes (ARGs) into the environment. However, tracking the origin of environmental ARGs is challenging. We found that widespread human fecal contamination was strongly correlated with ARGs in surface waters across a multi-use watershed. Remarkably, our novel synthesis of qPCR and geospatial data showed human fecal and ARG contamination were highly disseminated and associated with sewer network density and septic system age (non-point sources of contaminants) rather than wastewater reclamation outfalls or other point sources. These data suggest aging wastewater transmission networks and septic systems are an important contamination source and that aggressive investment to repair or replace aging and obsolete wastewater infrastructure is critical for mitigating environmental antibiotic resistance dissemination.

Author contributions

EAO, JRW, CRJ, JGF, and EKL designed the experiment; JRW, MET, and EAO collected and extracted DNA; JRW and JD ran qPCR analyses; JMBM and KAC performed geospatial analyses; JD analyzed data, with input from all authors; JD and JRW wrote the manuscript, with input from all authors. All authors approve of the final article.

Supporting information

Supporting Information File 1: Additional figures and detailed statistical analyses.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data are available on FigShare (doi: 10.6084/m9.figshare.20009042).

Research Data

Raw data are available on FigShare (doi: 10.6084/m9.figshare.20009042), including complete datasets for qPCR data, geospatial data, and correlations. Site-linked sewer and septic data are withheld due to potential privacy concerns.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2022.118853.

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