A Scenic River in Rural Ohio Shows Elevated Antibiotic Resistance Genes, Including *Acinetobacter* Tetracycline and Macrolide Resistance, Downstream of Wastewater Treatment Plant Effluent

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ABSTRACT

Rivers in rural areas receive continual influx of wastewater carrying antibiotics originally administered to humans and livestock. The entry of antibiotic resistance genes (ARGs) into aquatic systems has been documented for large municipal wastewater treatment plants, but there is less study of the impact of smaller plants that are situated on small rural rivers. We sampled water metagenomes for ARG and taxa composition from the Kokosing State Scenic River, a small rural river in Knox County, Ohio. Samples were obtained upstream, a few meters downstream, and 6 km downstream from the effluent release of the Mount Vernon wastewater treatment plant (WWTP). Through all seasons, the metagenome just downstream of the WWTP effluent showed a substantial elevation of at least 15 different ARGs, including 6 ARGs from *Acinetobacter baumannii* such as *msrE, mphE* (macrolide resistance) and *tet*(39) (tetracycline resistance). The ARGs most prevalent near the effluent pipe persisted 6 km downstream. The taxa distribution near the effluent showed elevation of *Acinetobacter* species as well as gut-associated taxa, Bacteroides and Firmicutes. The ARG levels and taxa prevalence showed little dependence on seasonal chlorination of effluent. Nitrogen and phosphorus were elevated near the effluent pipe but had no consistent effect on ARG levels. We show that in a rural river microbiome, year-round wastewater effluent substantially elevates ARGs including those of multidrug-resistant *A. baumanii.*

IMPORTANCE

Antibiotic resistance is a growing problem worldwide, with frequent transmission between pathogens and environmental organisms. Rural rivers support recreational use by people unaware of inputs from treated wastewater. In our study, the river water proximal to wastewater effluent shows increased prevalence of multidrug-resistant *Acinetobacter baumanii*, an opportunistic

pathogen of concern for hospitals but also widespread in natural environments. Our work highlights the importance of wastewater effluent in management of environmental antibiotic resistance.

INTRODUCTION

Environmental antibiotic resistance increasingly threatens global public health (1–3). Antibiotics from clinical use and livestock husbandry promote the development of resistant bacteria, and they readily pollute urban and rural waterways (4–6). Even sublethal concentrations of antimicrobial drugs select for resistance (7). Antibiotic resistance genes (ARGs) that enter environmental microbial communities have the potential to transfer to pathogenic bacteria (8). A major source of ARGs and antibiotics in aquatic systems is the effluent of wastewater treatment plants (WWTPs) (9–11). Wastewater treatment may actually select for increased antibiotic resistance of potential pathogens such as *Acinetobacter* species (12,13). It is important to understand the potential of WWTP to transfer ARGs as well as resistant microbes into rural streams, where they may disturb autochthonous microbial communities and spread drug resistance to human microbiomes. We investigated the impact of WWTP effluent on the taxa distribution and ARG counts in the Kokosing State Scenic River, a rural river designated "scenic" by the Ohio Department of Natural Resources.

The river microbiome may be affected by WWTP effluent in various ways: by elevation of phosphorus, nitrogen, and organic nutrients; by introduction of exogenous microbes; and by introduction of DNA including ARGs. The WWTP in our study chlorinates effluent only during the months of May through October, so we compared both conditions. While chlorination effectively decreases bacterial communities, it does not fully remove resistance genes from effluent. Some studies show partial decrease of ARGs by chlorine (14,15), whereas others show that chlorination may increase the effluent content of ARGs and promote their conjugative transfer (16,17). Various stress conditions in the WWTP can co-select antibiotic resistances and virulence properties (18). The release of heavy metals, antibiotics, and other compounds into

receiving rivers (such as nitrogen, phosphate, or ammonia) further propagates resistance by selecting for multidrug efflux pumps (19–21).

The establishment of anthropogenic resistance in environmental microbial communities can be controlled when municipalities reduce antibiotic usage (22). Therefore, understanding the impact of ARG pollutants on rural river resistomes is important for understanding the lasting potential of resistance in the environment. River resistomes offer the opportunity for surveillance of opportunistic pathogens that move between environment and human host, such as the ESKAPE pathogen *Acinetobacter baumanii* (23–25). The ESKAPE acronym comprises six leading hospital-acquired pathogens with multidrug resistance (26). While *A. baumanii* is known for hospital transmission, recent reports indicate community acquisition of strains that carry ARGs on plasmids (27,28). In the Kokosing we examined evidence for *Acinetobacter* ARGs such as *tet(39)* (29,30) and *msrE, mphE* (31).

To understand how WWTP effluent might alter rural river microbial communities, we sampled sites upstream, midstream and downstream of the effluent release of the Mount Vernon WWTP on the Kokosing River. The Kokosing river in east-central Ohio, USA, flows 92 km into the Walhonding River, a part of the watershed of the Mississippi River (32). Our study focused on a segment of the Kokosing in Knox County, proximal to the WWTP that serves the City of Mount Vernon (pop. 17,000). The Mount Vernon catchment includes surrounding suburban and rural homes as well as the Knox Community Hospital.

MATERIALS AND METHODS

Water sampling and metadata. Water samples were obtained at three sites on the Kokosing river (**Fig. 1**). The Upstream site (coordinates 40.38368, -82.47042) lies approximately 1.5 km upstream of the WWTP effluent discharge. The Midstream site, nearest the WWTP (40.378007, -82.467822) is located 9 m downstream of the effluent release pipe. The downstream site (40.376038, -82.40346) lies approximately 6 km downstream of the WWTP. The next nearest site where wastewater enters the Kokosing is the Fredericktown WWTP, approximately 1.6 km upstream of the Mount Vernon WWTP. The three river sites were sampled by identical procedures at six dates of the year. Water samples were collected on October 27, November 3, and December 3, 2019; and on April 13, May 28, and June 25, 2020. The WWTP effluent undergoes chlorination before discharge only from May 1st through October 31st; thus, only the October, May and June samples occurred during the time that effluent was chlorinated.

On each sampling date, the three sites were sampled within a 2-h period. At each site, 400 ml water was collected from the river using a dipper and sealed in a sterile bottle. The water was transported to a lab for analysis. Within 24 h of each sample date, three 100-ml samples were vacuum-filtered through a sterile 0.22-micron filter, 45 mm in diameter. Filter paper was folded using sterile forceps and deposited in centrifuge tubes which were then frozen at -80°C to preserve microbial DNA. Water was analyzed using a portable spectrophotometer, including pH, temperature, DO, nitrogen concentration and phosphorus concentration (**Supplemental Table S1**, Supporting Information). We chemically assayed nitrogen, phosphate and ammonia concentrations using a Hach® DR900 Multiparameter Portable Colorimeter.

DNA isolation and sequencing. Metagenomic DNA was isolated using a ZymoBIOMICS DNA Miniprep Kit. Each filter was cut into small pieces and transferred to a ZR BashingBead Lysis Tube. 650 µl ZymoBIOMICS Lysis Solution was added, and all tubes were processed on a Vortex Genie 2 for 40 min. The remainder of the preparation was performed according to the manufacturer's protocol. Shotgun sequencing of DNA was performed by Admera Health (www.admerahealth.com). Libraries for sequencing were prepared using Illumina's Nextera XT DNA Library Preparation Kit, following manufacturer's instructions. Final libraries were then pooled and sequenced on Illumina HiSeq X sequencer for 150-bp read length in paired-end mode, with an output of 40 million reads per sample.

ARG marker analysis. Sequence reads were analyzed for ARG marker hits using ShortBRED, a computational pipeline from Huttenhower Lab (33). ShortBRED-Identify was used to create a database of short marker peptides specific to ARGs from the Comprehensive Antibiotic Desistance database (CARD) (34). The CARD database of ARGs was grouped in families and short consensus peptides were identified. The set of peptides was then filtered against the Univeral Protein Database UNIREF90 (https://www.uniprot.org/uniref/) (data accessed October 23, 2019). This database was used to eliminate markers that match any sequences outside a specific ARG. One additional marker, ARO_3002930 (vanRO, *Rhodococcus_hoagii*) was removed from the marker set because inspection showed it lacked specificity. The final list of markers (ShortBRED-2019) is presented in **Supplemental Table S2**.

The ShortBRED-2019 marker list was used to screen metagenomic reads from each of the three river sites, from six sampling dates (**Supplemental Table S3**). Total read counts per sample were determined using Trimmomatic (35) (**Supplemental Table S4**).

Taxa profiles. The microbial taxa were profiled using the Huttenhower lab pipeline MetaPhlAn2 (Metagenomic Phylogenetic Analysis) MetaPhlAn2 (36,37). MetaPhlAn2 assigns metagenomic reads to taxa using a set of clade-specific marker genes. Taxa were grouped at the levels of phylum, class, order, family, and genus (**Supplemental Table S5**).

Data Analysis. To generate an ARG heatmap from the ShortBRED data, we employed R Studio ® 1.3.073. For One-Way ANOVA analysis, we used JMP ® 14.2.0. One-Way ANOVA was used to analyze the significance of ARG and taxa variances among the sites. ARG hits and metadata were correlated by the Spearman rank correlation using R (**Supplemental Table S6**).

Data submission. For all DNA sequences, FASTQ files were submitted to NCBI, SRA accession number PRJNA706754.

RESULTS

ARGs are elevated downstream of the WWTP effluent. We sought to determine how the ARG distribution of the Kokosing River microbiomes was affected by the effluent from the Mount Vernon WWTP. Microbial samples were obtained from three sites on the Kokosing River, designated Upstream (1.5 km upstream of the WWTP), Midstream (just below the effluent pipe) and Downstream (6 km downstream of the effluent pipe) (**Fig. 1**). From all sites the metagenomic DNA sequences were analyzed for ARG prevalence using the ShortBRED pipeline (33) applied to the CARD database (34). For each marker, the numbers of read hits were summed across all samples and dates, and the markers were ranked according to total hits (**Supplemental Table S3**). Results for the top 60 scoring markers are presented as a heat map (**Fig. 2**).

Most of the top-scoring ARGs were elevated in the Midstream samples, compared to samples from either Upstream or Downstream. The elevated ARGs include resistance determinants from several organisms that are of clinical concern. Most striking, six of the abundant ARGs come from the ESKAPE pathogen *Acinetobacter baumanii* and related strains: *msrE*, *mphE*, *tet(39)*, CfxA6, *oxa280*, and *aadA4* (23–25). The three top-ranked ARGs (*msrE*, *mphE*, *tet(39)*) are found together on *A. baumanii* plasmid pS30-1 (31). Overall, the four top *A. baumanii* ARGs account for 37% of the total ARG hits found.

We tested whether the Downstream samples show evidence of carryover from the Midstream site. First, the numbers of ARG hit reads were re-sorted by Midstream site ARG totals (**Table 1**). For each of the top-ranked Midstream ARGs, we present the difference in ARG hits between Upstream and Downstream. The top 27 most abundant Midstream ARGs, including those from *Acinetobacter*, all show higher numbers at Downstream site than Upstream.

The overall percentage of reads that matched ARG markers ranged from 0.0015-0.0052% for Midstream samples, and from 0.0002-0.0010% for Upstream and Downstream samples. These numbers indicate roughly 5-fold elevation of ARG hits in the Midstream, compared to the other two sites. We considered the possible effect of sample size, that is, whether the ARG hit numbers reflect the number of reads in our samples (**Supplemental Table S4**). The read counts from individual samples deviated less than 20% from the mean. There was no significant difference in read numbers amongst the three collection sites Upstream, Midstream, and Downstream. Thus, the elevated number of ARGs near the effluent pipe was independent of the number of sequenced reads per sample.

Taxa profiles associated with WWTP effluent. We investigated whether the elevation of ARGs by the WWTP was associated with specific microbial taxa. The taxa structure of our river metagenomes was determined using the pipeline MetaPhlAn2 (36). The distribution of major bacterial phyla and classes in our samples is shown in **Fig. 3A**, with p-values for Wilcoxon rank sum test (**Fig. 4A**). The genus *Acinetobacter* showed a striking prevalence in the Midstream, accounting for as high as 30% of predicted organisms (June sample, Midstream); and in some months elevated levels persisted downstream (December and April). Given that *A. baumanii* ARGs accounted for 37% of the total ARG hits, these results would be consistent with a high prevalence of multidrug-resistant *A. baumanni* associated with the WWTP plant effluent.

The Midstream site showed proportions of several major taxa significantly higher than those Upstream (**Fig. 3A**). The taxa with greater abundance include Bacteroidetes (p=0.002), Epsilonproteobacteria (p=0.002), Gammaproteobacteria (p=0.002) and Firmicutes (p=0.005). Downstream taxa appeared largely similar to those Upstream, with the exception of elevated

abundance of Bacteroidetes (p=0.015). These four taxa are consistent with a human fecal source, although they were present during the period of effluent chlorination as well as during absence of chlorination. The Upstream and Downstream sites showed higher proportions of Actinobacteria relative to the Midstream. Alphaproteobacteria and Betaproteobacteria showed high prevalence across all three sites. High prevalence of Actinobacteria and Betaproteobacteria are consistent with metagenomic studies of freshwater oligotrophic lakes and rivers (38).

We considered whether the Midstream elevated ARGs might be associated with bacterial clades that were enriched in Midstream samples. A Spearman rank correlation was performed comparing ARG hits with the major taxa identified (**Fig. 4C**). ARGS were categorized as "Top 60" and "below 60" based on overall rank prevalence (**Fig. 2** and **Supplemental Table S3**). The "Top 60" were those ARG classes showing relative elevation at the Midstream site near the WWTP effluent, whereas ARGs "Below 60" (ranked below the top 60 ARGs) more likely represent autochthonous genes commonly found in a relatively undisturbed river ecosystem. The number of ARG hits at Midstream and Downstream showed a positive correlation with Firmicutes and Epsilonproteobacteria, taxa that might be expected to arise from the WWTP effluent. Negative correlations were seen between ARGs and Betaproteobacteria, which are most likely native to the river.

If the source of "top 60" ARGs is the WWTP, are they carried by the genomes of effluent bacteria, or do they enter the river in the form of environmental DNA? The answer is unclear from our data. However, the occurrence of effluent chlorination (during the months of November, December and April) shows no significant effect on the Midstream taxa profiles (**Fig 3A**). If live bacteria are responsible for ARGs elevation, significant numbers must be surviving chlorination.

Nitrate, phosphate and ammonia levels show no correlation with elevated ARGs. The Midstream water samples showed elevated levels of nitrate, phosphate and ammonia (**Fig. 5** and **Supplemental Table S1**). We therefore looked for possible correlations between water chemistry and ARG prevalence. Spearman rank correlations were performed for ARG levels and various chemical and physical factors (**Supplemental Table S5**). Correlations were run separately for the sums of Top 60 ARG hits and for the sums of Below 60 ARG hits. We hypothesized that the top 60 ARGs are dominated by the WWTP effluent and would therefore show stronger correlations with the Midstream chemistry.

In fact, the nitrate, phosphate and ammonia levels showed no consistent correlations with ARGs, either Top 60 or Below 60. This finding suggests that, despite the elevation of these minerals near the effluent pipe, nitrogen and phosphorus loading are not responsible for ARGs elevation.

ARG numbers increased with pH and temperature. The strongest ARG correlations we did find for water chemistry were for pH and temperature (**Supplemental Table S5**). At Midstream and Downstream sites, pH showed a strong positive correlations with ARG hits, particularly the Below 60 ARGs. These results suggest that low pH might select against ARGs that commonly occur in metagenomes of the undisturbed river. In laboratory evolution experiments on *Escherichia coli*, low pH and membrane-permeant aromatic acids select for loss of drug resistance genes (39,40).

Temperature showed a strongly negative correlation with ARG levels, particularly those Below 60. This finding would be consistent with high-temperature selection against ARGs commonly found in the river community.

DISCUSSION

The footprint of the WWTP effluent release was evident across our data, including shifts in ARG prevalence (**Fig. 2, Table 1**), microbial community taxa distribution (**Fig. 3**), and chemical indicators (**Fig. 5**). The top three ARGs commonly occur together on *A. baumanii* plasmid pS30-1 (31); and *A. baumanii* showed high prevalence near the WWTP effluent (**Fig. 3B**). It is possible that the multidrug-resistant *A. baumanii* actually comes from the WWTP. Wastewater treatment is known to increase the prevalence of multidrug resistance in *A. baumanii* from influent to the final effluent (12). Thus, an effect of the wastewater effluent could be to increase community exposure to drug-resistant strains of this ESKAPE pathogen.

Rural rivers have substantial economic and cultural significance for local communities, as examplified by the state "scenic" designation and promotion of recreational activities. The public is rarely aware of the potential impact of WWTP ARG exposure, despite the common presence of WWTP plants along rural rivers. For example, a few km upstream of the Mount Vernon plant is the Fredericktown WWTP; and just downstream of our sampled sites in Gambier another small WWTP releases effluent to the Kokosing.

We found evidence that detectable levels of ARGs persist in the river microbial community several kilometers past the effluent pipe. The Downstream site exhibited higher ARG counts than Upstream for the top 27 ARGs elevated at Midstream (**Table 1**). Thus, WWTP-associated ARGs persist in the environment at least 6 km downstream. These ARGs might become established in the river microbial resistome and could propagate to pathogenic bacteria in the future.

The WWTP-proximal site also showed substantial alteration of overall taxa distributions, such as increased prevalence of Bacteroidetes and Firmicutes, findings consistent with previous

study (35). There is need for future study regarding efficient methods of ARG control from WWTP, such as limiting the concentration of heavy metals in the plant to prevent co-selection of multi-drug efflux pumps (19,42). Better awareness of the consequences of WWTP-effluent release into rivers will improve our ability to sustain healthy microbial communities in our freshwater systems.

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TABLES

Table 1. Top-ranked Midstream ARG hits: Difference in hit numbers between Downstream and Upstream Sample Sites*

Mid Rank	ARG Family	Resistome where Found or Prevalent	Reads: Down - Up
1	*msrE*	*Acinetobacter baumannii*	60
2	*mphE*	*Acinetobacter baumannii*	64
3	*tet(39)*	*Acinetobacter baumannii*	51
4	CfxA6	*Acinetobacter baumannii*	70
5	OXA-256	*Enterobacter cloacae*	28
6	Mef(En2)	*Bacteroides fragilis*	36
7	AAC(3)-VIIa	*Streptomyces rimosus*	34
8	OXA-5	*Pseudomonas aeruginosa*	9
9	QnrS6	*Aeromonas hydrophila*	25
10	OXA-226	*Pseudomonas aeruginosa*	16
11	ANT(4_)-Ib	*Staphylococcus aureus*	38
12	*mtrA*	*Mycobacterium tuberculosis*	8
13	*sul1*	*Vibrio fluvialis*	12
14	OXA-280	*Acinetobacter johnsonii*	11
15	*tetE*	*Escherichia coli*	4
16	EreA2	*Providencia stuartii*	3
17	*mefC*	*Photobacterium damselae*	7
18	*tetQ*	*Bacteroides fragilis*	9
19	OXA-46	*Pseudomonas aeruginosa*	9
20	MOX-5	*Aeromonas caviae*	11
21	CblA-1	*Bacteroides uniformis*	2
22	ErmB	*Enterococcus faecium*	17
23	*(aadA4)*	*Acinetobacter baumannii*	1
24	*mphG*	*Photobacterium damselae*	6
25	ErmG	*Bacteroides thetaiotaomicron*	4
26	*dfrF*	*Enterococcus faecalis*	2
27	ErmF	*Bacteroides fragilis*	14

***ARGs shown represent the top 30 most abundant ARGs from Midstream sites. Values in right-hand column indicate the difference between total Downstream reads and total Upstream reads that match the marker shown.

FIGURES

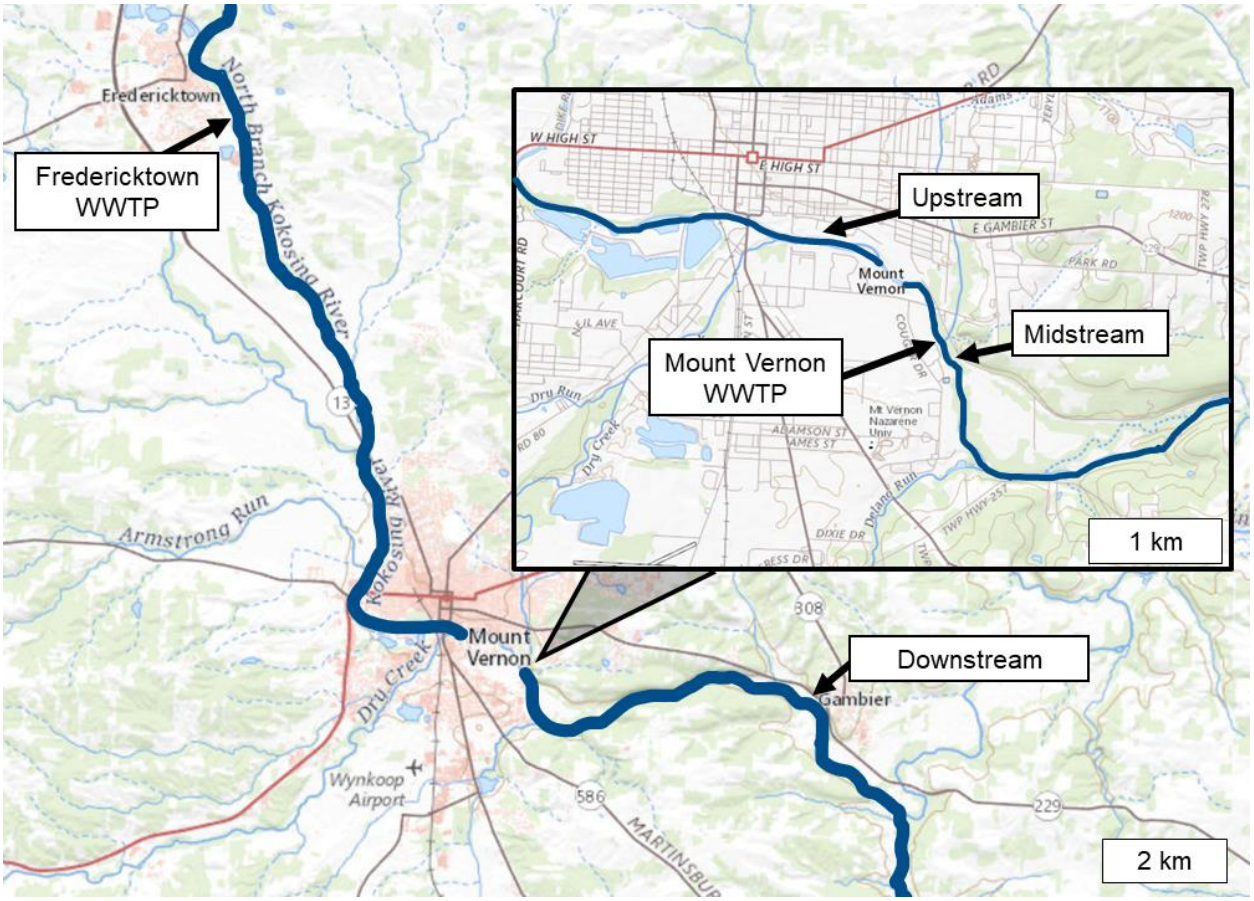
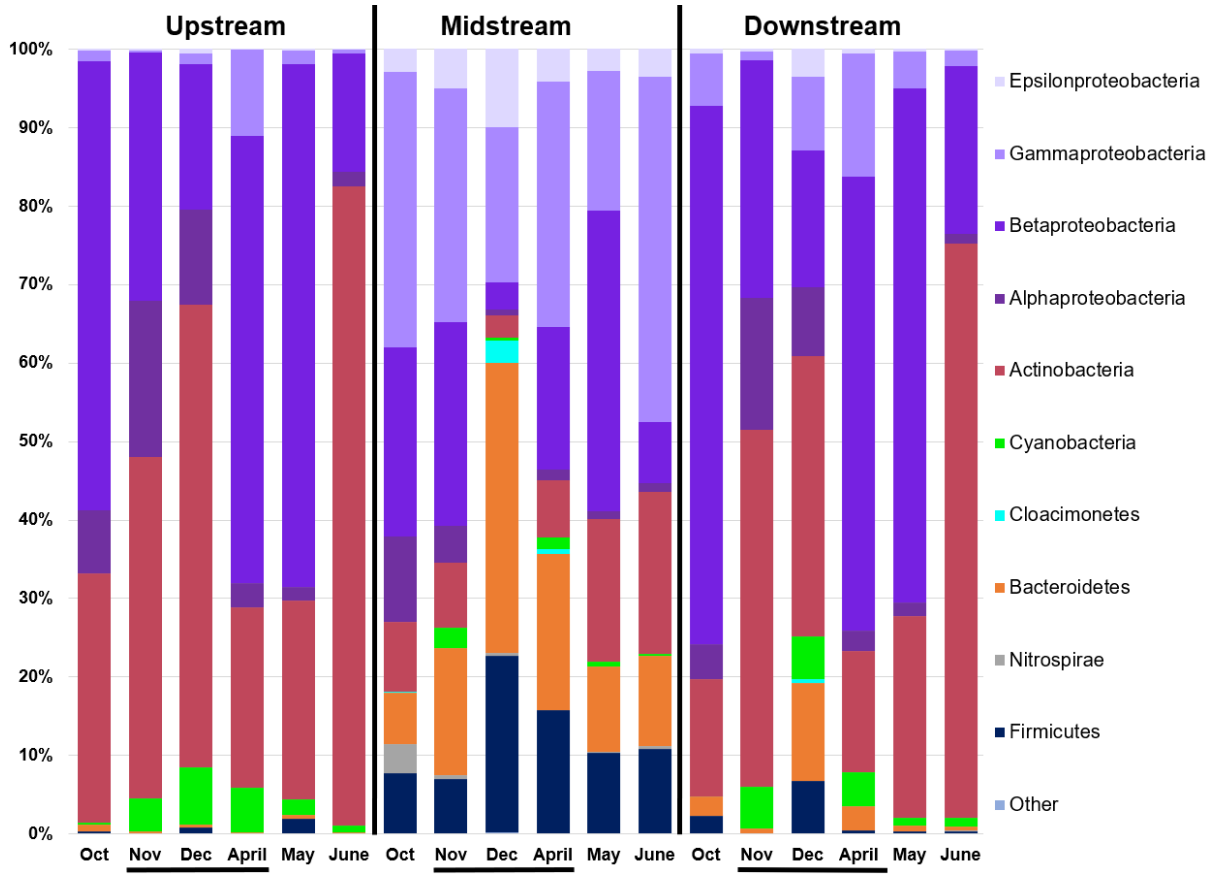


Figure 1. Map of water sampling sites on the Kokosing River. The Upstream site is 1 km upstream of the Mount Vernon City Wastewater Treatment Plant (WWTP). Midstream site is located 9 m downstream of the WWTP. Downstream site is 6 km downstream of the WWTP. Map was generated using the National Wild and Scenic Rivers System (2021).

	ARG	Taxon of Origin or Prevalence	October			November			December			April			May			June			Total
		Family	Up	Mid	Down	Up	Mid	Down	Up	Mid	Down	Up	Mid	Down	Up	Mid	Down	Up	Mid	Down	
1	*msrE*	*Acinetobacter baumannii*	0	60	5	0	97	4	8	244	33	2	210	6	0	110	2	2	1011	22	1816
2	*mphE*	*Acinetobacter baumannii*	0	56	6	0	99	4	2	174	29	2	178	9	0	95	0	0	964	20	1638
3	*tet(39)*	*Acinetobacter baumannii*	0	26	6	0	100	2	3	101	30	0	83	11	0	124	2	0	154	3	645
4	CfxA6	*Acinetobacter baumannii*	0	21	10	0	36	0	0	207	50	0	63	6	2	48	2	0	85	4	534
5	AAC(3)-VIIa	*Streptomyces rimosus*	10	119	43	9	40	20	11	44	8	13	7	13	11	9	5	12	13	11	398
6	ANT(4)	*Staphylococcus aureus*	0	73	98	0	0	0	17	21	11	0	0	1	61	80	0	1	1	7	371
7	OXA-256	*Enterobacter cloacae*	3	65	16	0	48	5	2	97	11	0	29	2	3	32	2	0	54	0	369
8	Mef(En2)	*Bacteroides fragilis*	0	11	2	0	17	0	2	88	25	0	42	1	0	23	10	0	52	0	273
9	OXA-5	*Pseudomonas aeruginosa*	0	28	1	0	50	0	3	32	10	0	41	0	0	15	1	0	62	0	243
10	QnrS6	*Aeromonas hydrophila*	0	16	0	0	29	3	0	65	16	1	33	4	0	27	0	0	29	3	226
11	OXA-226	*Pseudomonas aeruginosa*	0	13	7	0	22	0	2	73	10	0	13	0	0	19	1	0	51	0	211
12	*mtrA*	*Mycobacterium tuberculosis*	0	14	2	2	12	3	3	20	1	0	24	0	0	9	2	3	95	8	198
13	*sul1*	*Vibrio fluvialis*	0	42	2	0	12	0	1	53	6	0	11	3	0	14	2	2	40	2	190
14	OXA-280	*Acinetobacter johnsonii*	0	7	0	0	23	1	0	34	10	0	8	0	1	21	0	0	67	1	173
15	*tet(E)*	*Escherichia coli*	0	2	0	0	32	2	1	40	5	2	25	0	0	1	0	0	43	0	153
16	*mefC*	*Photobacterium damselae*	0	14	2	0	32	0	0	22	4	0	8	0	0	20	1	0	31	0	134
17	EreA2	*Providencia stuartii*	0	58	0	0	32	2	0	13	1	0	0	0	0	5	0	0	19	0	130
18	ACT-35	*Enterobacter cloacae*	3	5	6	8	4	9	8	6	16	6	4	9	13	6	1	5	6	4	119
19	OXA-156	*Pandoraea pulmonicola*	3	10	4	2	0	7	3	14	7	12	9	10	3	13	5	1	3	7	113
20	*tetQ*	*Bacteroides fragilis*	0	0	0	0	6	0	0	59	7	0	11	2	0	3	0	0	14	0	102
21	OXA-46	*Pseudomonas aeruginosa*	0	12	0	0	16	0	0	29	4	0	12	4	1	4	0	0	17	2	101
22	ErmB	*Enterococcus faecium*	0	1	0	0	2	0	0	34	17	0	17	0	1	6	0	0	15	1	94
23	MOX-5	*Aeromonas caviae*	0	6	1	0	8	0	1	6	8	0	10	0	0	11	2	0	37	1	91
24	Erm(O)-Irm	*Streptomyces lividans*	0	2	0	3	3	3	2	2	3	11	9	3	2	18	1	9	9	3	83
25	CblA-1	*Bacteroides uniformis*	0	1	0	0	7	0	0	31	1	0	11	1	0	13	0	0	15	0	80
26	SHV-100	*Klebsiella pneumoniae*	2	13	7	2	2	3	2	4	1	4	5	4	5	7	2	3	10	2	78
27	*mphG*	*Photobacterium damselae*	0	6	2	0	10	0	0	19	3	0	7	0	0	6	0	0	21	1	75
28	OXA-443	*Ralstonia mannitolilytica*	5	3	4	5	7	5	2	15	0	0	5	1	3	4	4	2	7	3	75
29	*aadA4*	*Acinetobacter baumannii*	0	2	0	0	36	0	0	16	1	0	2	0	0	13	0	0	4	0	74
30	ErmF	*Bacteroides fragilis*	0	0	0	0	1	2	0	36	5	0	5	2	0	3	5	0	12	0	71
31	*cmlA5*	Gram-negative species	0	1	0	4	15	0	1	22	2	1	7	2	1	2	1	0	9	1	69
32	FOX-3	*Klebsiella oxytoca*	0	0	6	1	7	0	1	11	0	2	6	0	1	8	5	3	15	3	69
33	*ErmG*	*Bacteroides thetaiotaomicron*	0	1	0	0	5	1	0	38	3	0	14	0	0	3	0	0	2	0	67
34	*bcrC*	*Bacillus licheniformis*	0	3	0	6	3	0	2	29	2	1	5	0	0	2	0	2	8	1	64
35	OXA-137	*Brachyspira pilosicoli*	6	5	2	4	7	1	1	11	0	5	8	3	2	3	1	1	3	1	64
36	*dfrF*	*Enterococcus faecalis*	0	0	0	0	4	0	0	25	0	0	10	1	0	10	0	0	12	1	63
37	*tetW*	*Butyrivibrio fibrisolvens*	0	0	4	0	1	0	0	29	0	0	5	0	5	5	2	2	9	0	62
38	MOX-9	*Citrobacter freundii*	2	1	10	0	1	3	1	10	1	0	4	1	3	6	7	6	6	0	62
39	*mefB*	*Escherichia coli*	0	5	0	0	4	0	0	17	2	0	5	0	0	7	0	0	13	0	53
40	*qacH*	*Vibrio cholerae*	0	4	0	0	12	0	2	16	0	0	5	1	0	3	0	0	10	0	53
41	AER-1	*Aeromonas hydrophila*	0	8	2	0	5	0	0	13	1	0	4	0	0	0	0	0	17	0	50
42	ANT(3)	*Serratia marcescens*	0	2	1	0	7	0	0	26	0	0	1	0	0	7	0	0	4	1	49
43	EreD	*Riemerella anatipestifer*	0	2	0	0	14	0	0	7	0	0	0	0	2	8	0	0	14	2	49
44	*aadA27*	*Acinetobacter lwoffii*	0	2	0	0	7	2	4	8	1	3	3	5	0	5	0	3	4	2	49
45	GES-21	uncultured bacterium	2	1	0	0	1	0	0	19	0	0	9	0	0	1	0	0	15	0	48
46	*lnuC*	*Streptococcus agalactiae*	0	3	0	0	6	0	0	16	3	2	6	1	0	5	0	0	6	0	48
47	*sul2*	*Vibrio cholerae*	0	9	0	0	6	0	0	14	0	0	2	0	0	8	0	0	3	0	42
48	*mphA*	*Escherichia coli*	0	4	0	0	3	0	0	18	3	0	2	0	0	0	0	0	11	1	42
49	APH(6)-Id	*Pseudomonas aeruginosa*	0	6	0	0	4	0	0	16	2	0	5	0	0	0	0	0	8	0	41
50	OXA-31	*Pseudomonas aeruginosa*	0	7	0	0	7	0	2	13	1	0	1	0	0	5	0	0	5	0	41
51	*tet(L)*	*Geobacillus stearothermophilus*	0	12	7	0	0	0	0	2	2	0	0	0	9	9	0	0	0	0	41
52	IsaE	*Enterococcus faecalis*	0	0	1	0	3	0	0	14	2	0	7	0	0	1	0	0	12	0	40
53	*imiS*	*Aeromonas veronii*	0	0	0	0	5	0	2	14	4	0	3	0	0	5	0	0	4	0	37
54	*tet(40)*	*Clostroides* sp.	0	0	2	0	0	0	0	17	4	0	5	0	0	4	1	0	4	0	37
55	ANT(3)	*Escherichia coli*	0	5	1	0	0	2	0	9	2	0	4	1	0	5	0	0	4	1	34
56	OXA-45	*Pseudomonas aeruginosa*	0	2	0	0	1	0	0	16	0	0	1	0	0	5	0	0	7	0	32
57	*mel*	*Streptococcus pyogenes*	0	0	0	0	2	0	0	13	0	0	6	1	0	0	0	0	9	0	31
58	APH(3)-IIIa	*Campylobacter coli* CVM N29710	0	1	0	0	1	0	0	11	2	0	1	0	0	6	0	0	7	0	29
59	*tetX*	*Bacteroides fragilis*	0	1	0	0	1	0	0	6	1	0	5	0	0	4	1	0	8	0	27
60	*aads*	Transposon Tn4551	0	2	0	0	1	0	0	10	2	0	0	1	0	9	0	0	2	0	27

Figure 2. **Heatmap of relative abundance of top 60 ARG marker hits.** Read hit numbers are ranked in descending order by total hits across samples. Yellow represents highest abundance, cyan represents lowest abundance.

A.


Upstream
Midstream
Downstream
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Oct Nov Dec April May June Oct Nov Dec April May June Oct Nov Dec April May June
Epsilonproteobacteria
Gammaproteobacteria
Betaproteobacteria
Alphaproteobacteria
Actinobacteria
Cyanobacteria
Cloacimonetes
Bacteroidetes
Nitrospirae
Firmicutes
Other

B.

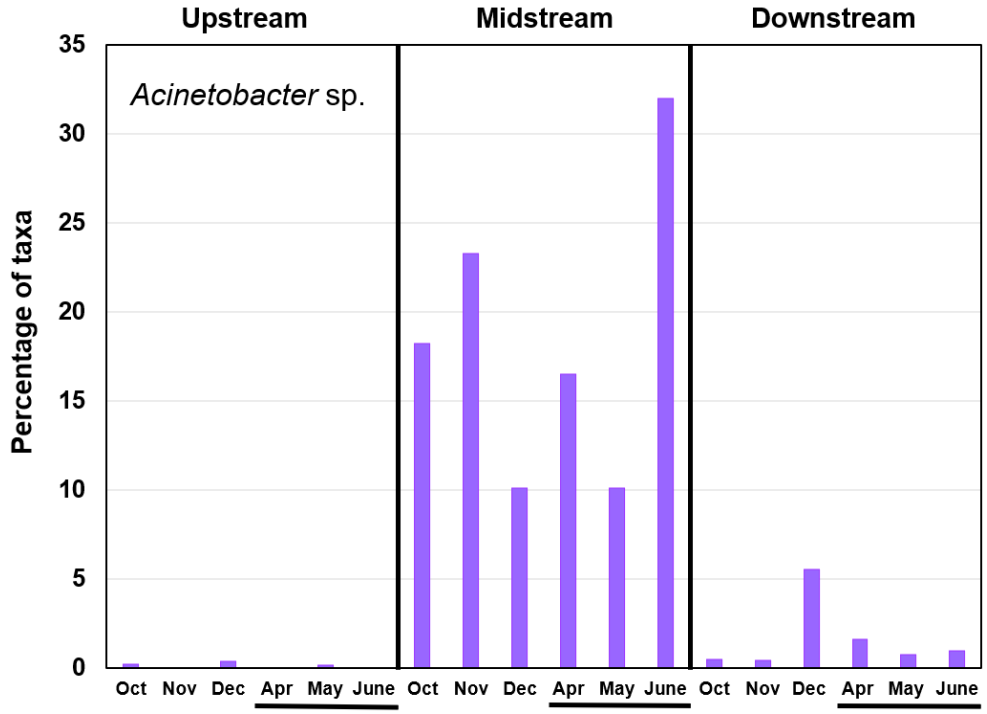

Upstream
Midstream
Downstream
Acinetobacter sp.
Percentage of taxa
35
30
25
20
15
10
5
0
Oct Nov Dec Apr May June Oct Nov Dec Apr May June Oct Nov Dec Apr May June

Figure 3. ARGs and bacterial taxa distribution across sampling sites and dates.

A. Percentages of phyla and of proteobacterial classes predicted by MetaPhlAn2. Taxa with prevalence too small to be quantified were grouped as "Other." Samples are sorted by site, then by sampling date. Horizontal black bars indicate dates when effluent was unchlorinated.

B. Percentage of *Acinetobacter* species predicted by MetaPhlAn2.

A.

	Alphaproteobacteria	Betaproteobacteria	Epsilonproteobacteria	Gammaproteobacteria	Actinobacteria	Bacteroidetes	Cloacimonetes	Cyanobacteria	Firmicutes
Midstream -- Downstream	0.18	0.093	0.015	0.002	0.026	0.015	0.608	0.199	0.002
Upstream -- Downstream	0.699	0.818	0.093	0.093	0.589	0.015	0.074	0.937	0.375
Midstream -- Upstream	0.093	0.132	0.002	0.002	0.004	0.002	0.074	0.093	0.005

B.

Taxa and ARG prevalence: Top 60 and Below 60	Upstream Top 60	Upstream Below 60	Midstream Top 60	Midstream Below 60	Downstream Top 60	Downstream Below 60
Firmicutes	0.55	0.84	0.43	0.71	0.89	0.83
Bacteroidetes	-0.20	0.31	0.66	0.49	0.49	0.77
Cyanobacteria	0.54	0.26	0.03	-0.49	-0.03	0.20
Actinobacteria	-0.20	-0.14	-0.09	-0.26	0.03	-0.31
Alphaproteobacteria	-0.49	-0.20	-0.54	-0.54	0.20	0.60
Betaproteobacteria	0.14	0.31	-0.77	-0.94	-0.37	-0.20
Gammaproteobacteria	0.66	0.60	0.20	0.09	0.43	0.60
Epsilonproteobacteria	-0.09	0.54	0.60	0.31	0.60	0.83

Figure 4. Taxa correlations across river sites.

A. Wilcoxon rank sum test p-values for comparison of taxa percentages at different sites, grouped across all dates. p-values ≤ 0.02 indicate significant differences (highlighted).

B. Spearman rank correlations between bacterial taxa and ARG abundance.

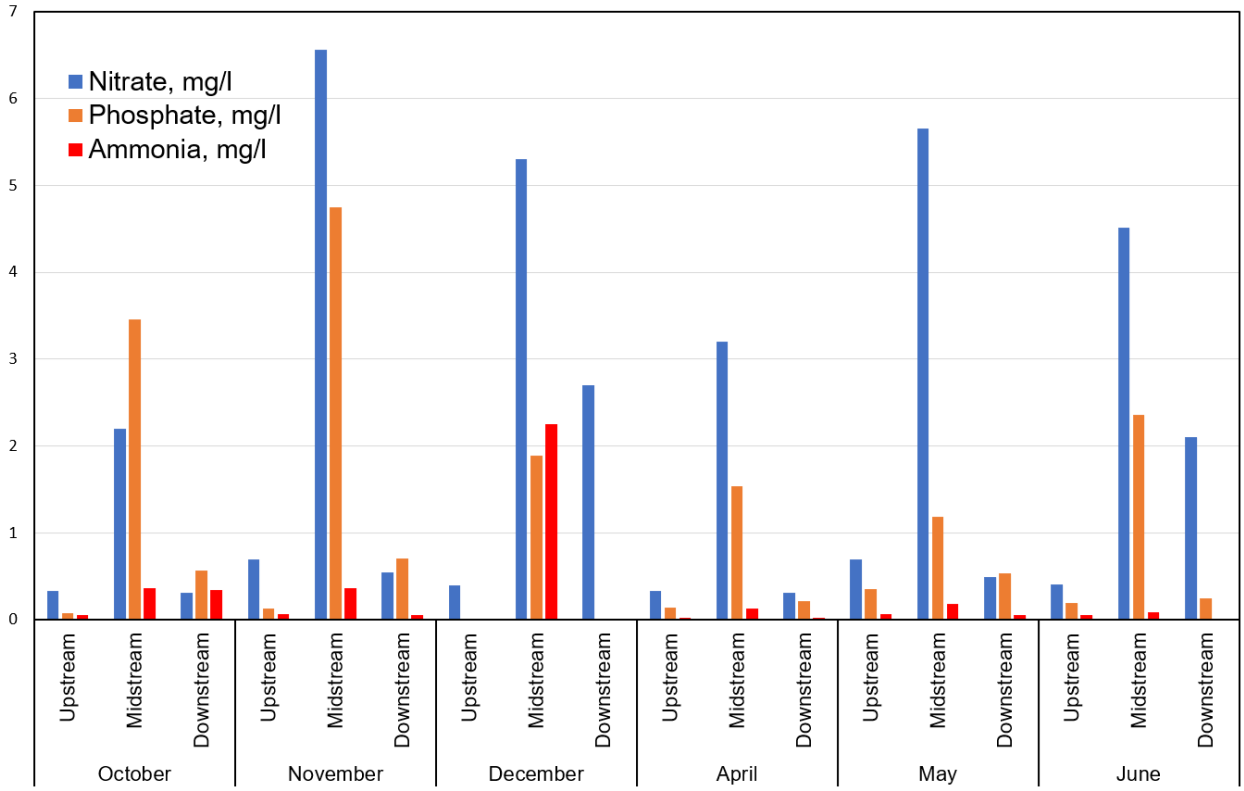


Figure 5. Nitrate, phosphate and ammonia concentrations across sites and dates. Concentrations of nitrate, phosphorus and ammonia were measured at each river site for each month. Full metadata are presented in **Supplemental Table S1**; and metadata correlations with ARG abundance in Supplemental **Table S6**.