Microbial Community Composition and Antibiotic Resistance Genes Within a North Carolina Urban Water System

*Kevin C. Lambirth[1,2], Matthew C. B. Tsilimigras[1], Anju Lulla[1], James Johnson[1], Abrar Al-Shaer[3], Orion Wynblatt[1], Shannon Sypolt, Cory Brouwer[1,2], Sandra Clinton[1], Olya Keen[1], Molly Redmond[1], Anthony Fodor[1], Cynthia Gibas[1]**

The University of North Carolina at Charlotte

9201 University City Boulevard, Charlotte NC, 28223[1]
150 N Research Campus Dr, Kannapolis NC, 28081[2]

The University of North Carolina at Chapel Hill

135 Dauer Drive, Chapel Hill NC, 27599[3]

Materials and Methods

Detection of antibiotic compounds using mass spectrometry

UPLC	
Column	ACQUITY UPLC BEH C18 1.7 µm analytical column (2.1 × 100 mm)
Column Temp (°C)	45 ± 0.5
Sample Manager Temp (°C)	4 ± 0.5
Gradient Conditions for ESI- mode	0-0.25 min (2% B), 0.25-4 min (2-99% B), 4-6 min (99% B), 6-6.01 min (99-2% B), 6.01-7 min (2% B)
Flow Rate (mL/min)	0.4
Quattro Premier MS	
Capillary (kV)	2.5
Sampling Cone (V)	30
Extraction Cone (V)	4.0
Source Temp (°C)	120
Desolvation Temp (°C)	350
Desolvation Gas Flow (L/Hr)	800
Cone Gas (L/Hr)	0

Compound	Parent (m/z)	Daughter (m/z)	Dwell (s)	Cone (V)	Collision (V)
Sulfamethoxazole	254.3	156.1	0.05	30	15
Trimethoprim	290.9	230.0	0.05	35	20
Ciprofloxacin	331.9	314.2	0.05	30	20
Cephalexin	347.9	158.0	0.05	35	10
Levofloxacin	361.9	318.2	0.05	30	20
Amoxicillin	365.9	114.2	0.05	20	15
Clindamycin	425.0	126.2	0.05	35	25
Doxycycline	445.0	428.2	0.05	30	15
Ertapenem	476.1	432.4	0.05	25	10
Azithromycin	749.3	158.1	0.05	40	30

Additional Results

Table S1

Genus	p Value
Peptostreptococcaceae	0.000120138
Afipia	0.000165926
Holospora	9.63E-05
Bppunalikevirus	0.000553161
Yualikevirus	0.000717512
Azoarcus	0.00101914
Legionellaceae	0.002209625
Methyloversatilis	0.006230025
Sphingobium	0.009322171
Rickettsiella	0.009056738
Myroides	0.017005632
Alicycliphilus	0.016215851
Burkholderia	0.025314864
Acinetobacter	0.025614406
Thauera	0.034797732
Riemerella	0.045239986

Figure S1

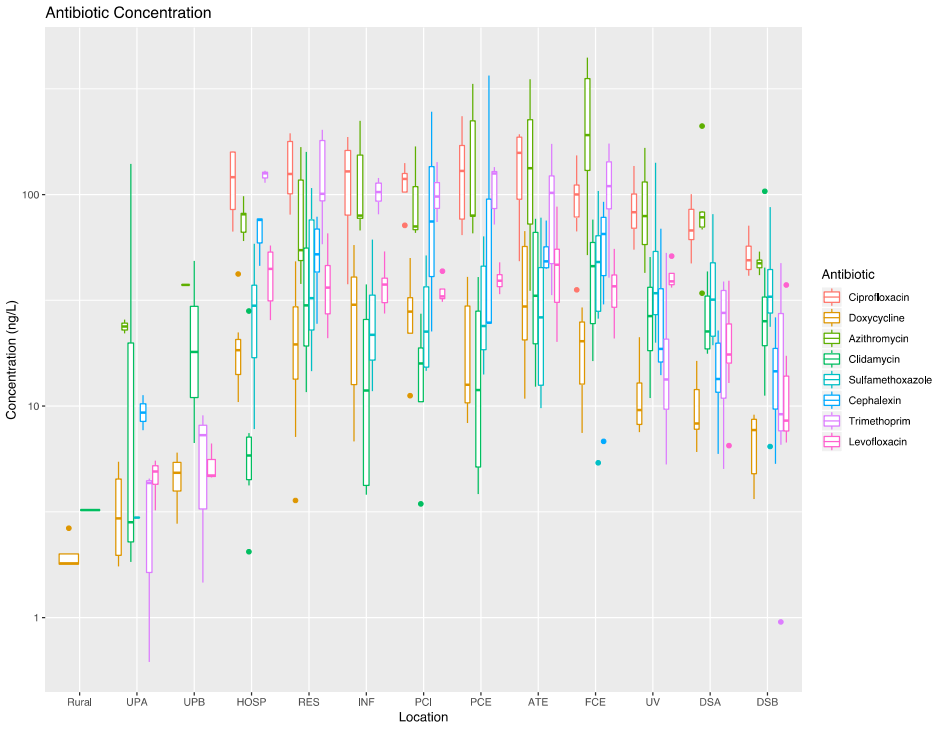

Figure S2

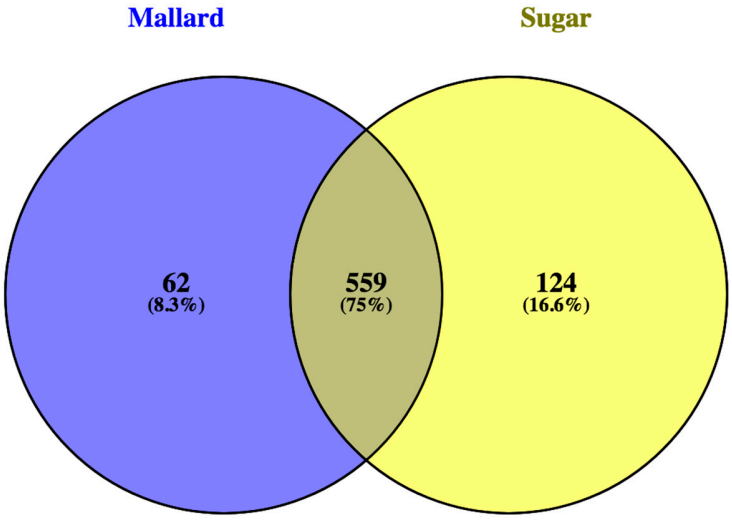

Figure S3

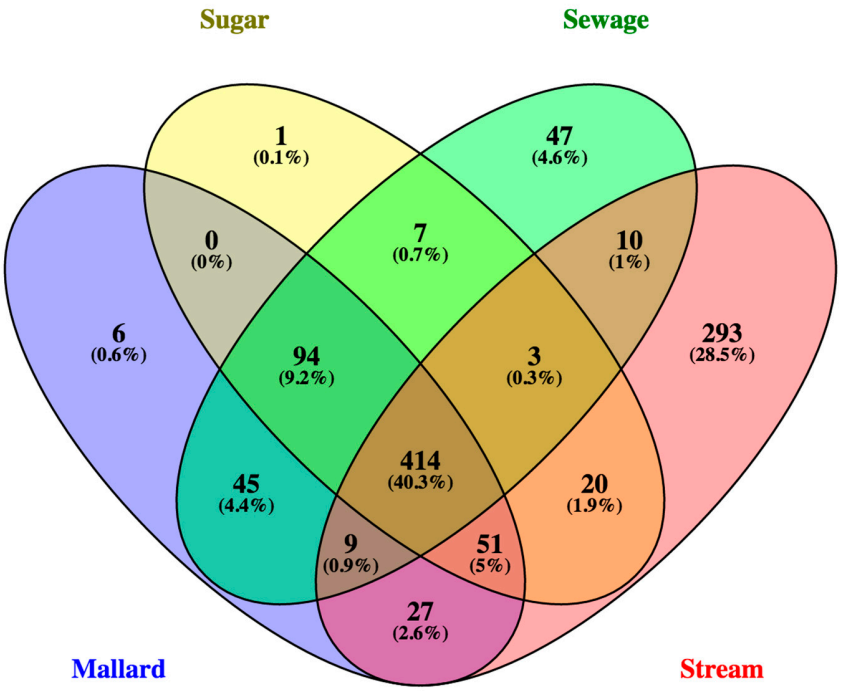
Sugar
Sewage
1
(0.1%)
47
(4.6%)
0
(0%)
7
(0.7%)
10
(1%)
6
(0.6%)
94
(9.2%)
3
(0.3%)
293
(28.5%)
414
(40.3%)
45
(4.4%)
20
(1.9%)
9
(0.9%)
51
(5%)
27
(2.6%)
Mallard
Stream

Figure S4

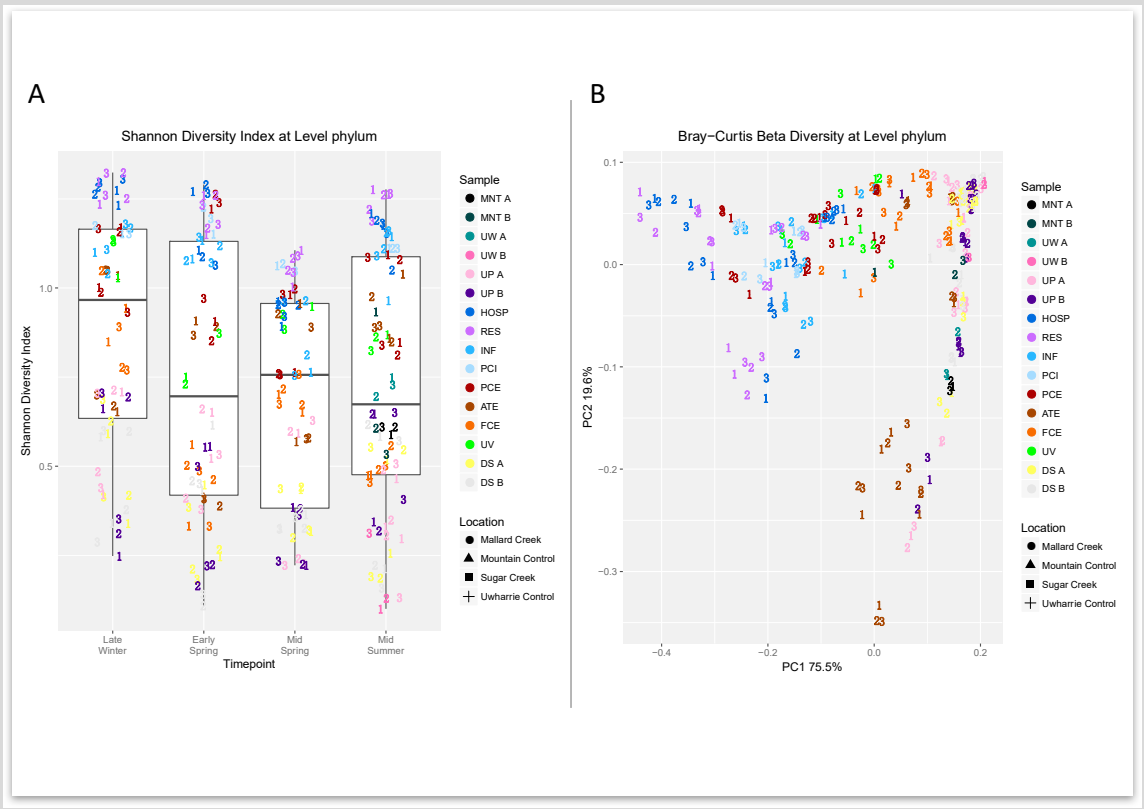
A
Shannon Diversity Index at Level phylum
Shannon Diversity Index
1.0
0.5
Late Winter
Early Spring
Mid Spring
Mid Summer
Timepoint
Sample
MNT A
MNT B
UW A
UW B
UP A
UP B
HOSP
RES
INF
PCI
PCE
ATE
FCE
UV
DS A
DS B
Location
Mallard Creek
Mountain Control
Sugar Creek
Uwharrie Control
B
Bray−Curtis Beta Diversity at Level phylum
PC2 19.6%
0.1
0.0
−0.1
−0.2
−0.3
−0.4
−0.2
0.0
0.2
PC1 75.5%
Sample
MNT A
MNT B
UW A
UW B
UP A
UP B
HOSP
RES
INF
PCI
PCE
ATE
FCE
UV
DS A
DS B
Location
Mallard Creek
Mountain Control
Sugar Creek
Uwharrie Control

Figure S5

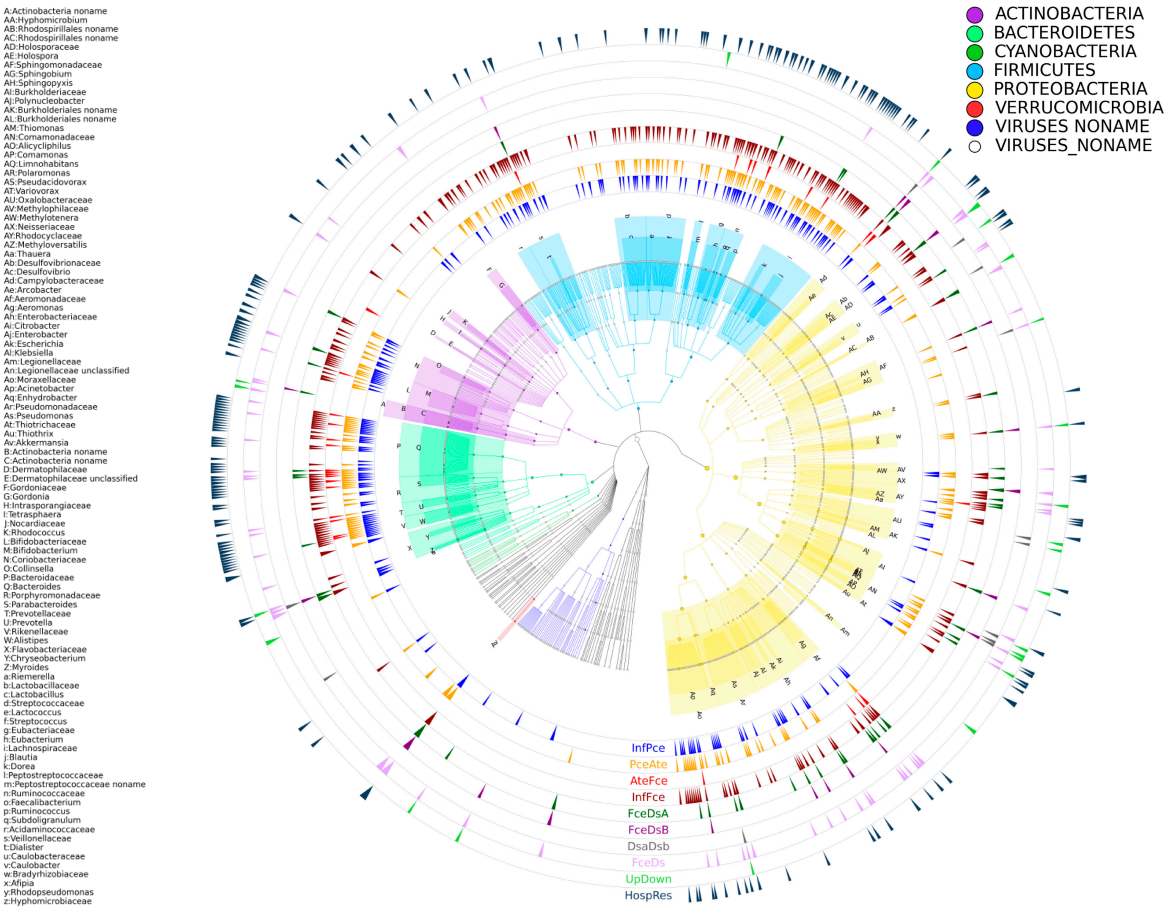
A:Actinobacteria noname
AA:Hyphomicrobium
AB:Rhodospirillales noname
AC:Rhodospirillales noname
AD:Holosporaceae
AE:Holospora
AF:Sphingomonadaceae
AG:Sphingobium
AH:Sphingopyxis
AI:Burkholderiaceae
AJ:Polynucleobacter
AK:Burkholderiales noname
AL:Burkholderiales noname
AM:Thiomonas
AN:Comamonadaceae
AO:Alicycliphilus
AP:Comamonas
AQ:Limnohabitans
AR:Polaromonas
AS:Pseudacidovorax
AT:Variovorax
AU:Oxalobacteraceae
AV:Methylophilaceae
AW:Methylotenera
AX:Neisseriaceae
AY:Rhodocyclaceae
AZ:Methyloversatilis
Aa:Thauera
Ab:Desulfovibrionaceae
Ac:Desulfovibrio
Ad:Campylobacteraceae
Ae:Arcobacter
Af:Aeromonadaceae
Ag:Aeromonas
Ah:Enterobacteriaceae
Ai:Citrobacter
Aj:Enterobacter
Ak:Escherichia
Al:Klebsiella
Am:Legionellaceae
An:Legionellaceae unclassified
Ao:Moraxellaceae
Ap:Acinetobacter
Aq:Enhydrobacter
Ar:Pseudomonadaceae
As:Pseudomonas
At:Thiotrichaceae
Au:Thiothrix
Av:Akkermansia
B:Actinobacteria noname
C:Actinobacteria noname
D:Dermatophilaceae
E:Dermatophilaceae unclassified
F:Gordoniaceae
G:Gordonia
H:Intrasporangiaceae
I:Tetrasphaera
J:Nocardiaceae
K:Rhodococcus
L:Bifidobacteriaceae
M:Bifidobacterium
N:Coriobacteriaceae
O:Collinsella
P:Bacteroidaceae
Q:Bacteroides
R:Porphyromonadaceae
S:Parabacteroides
T:Prevotellaceae
U:Prevotella
V:Rikenellaceae
W:Alistipes
X:Flavobacteriaceae
Y:Chryseobacterium
Z:Myroides
a:Riemerella
b:Lactobacillaceae
c:Lactobacillus
d:Streptococcaceae
e:Lactococcus
f:Streptococcus
g:Eubacteriaceae
h:Eubacterium
i:Lachnospiraceae
j:Blautia
k:Dorea
l:Peptostreptococcaceae
m:Peptostreptococcaceae noname
n:Ruminococcaceae
o:Faecalibacterium
p:Ruminococcus
q:Subdoligranulum
r:Acidaminococcaceae
s:Veillonellaceae
t:Dialister
u:Caulobacteraceae
v:Caulobacter
w:Bradyrhizobiaceae
x:Afipia
y:Rhodopseudomonas
z:Hyphomicrobiaceae
ACTINOBACTERIA
BACTEROIDETES
CYANOBACTERIA
FIRMICUTES
PROTEOBACTERIA
VERRUCOMICROBIA
VIRUSES NONAME
VIRUSES_NONAME
InfPce
PceAte
AteFce
InfFce
FceDsA
FceDsB
DsaDsb
FceDs
UpDown
HospRes

Figure S6

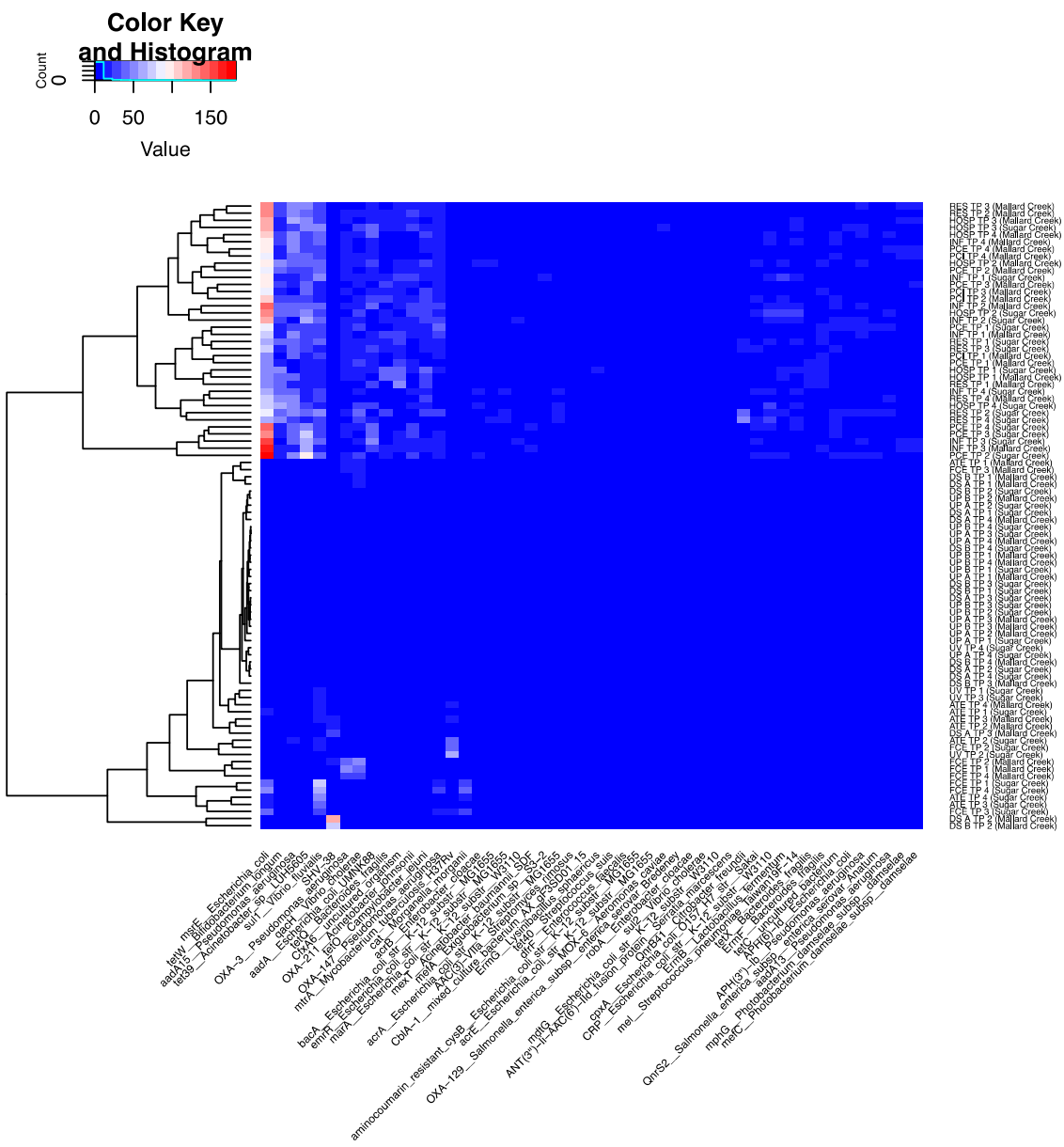

Figure S7

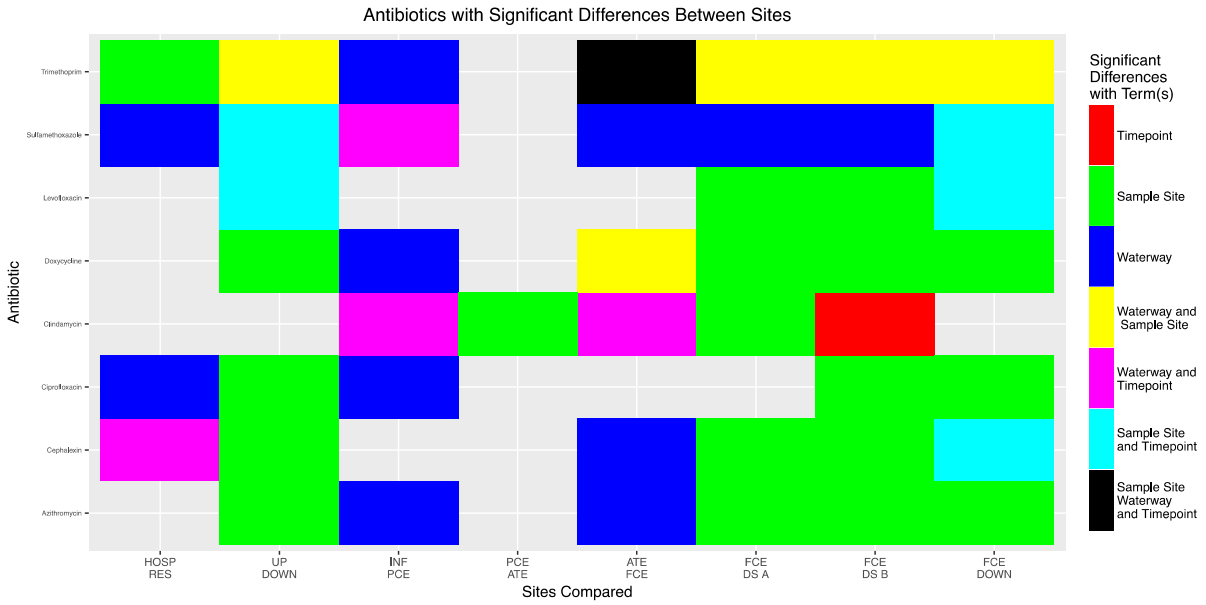
Antibiotics with Significant Differences Between Sites
Antibiotic
Trimethoprim
Sulfamethoxazole
Levofloxacin
Doxycycline
Clindamycin
Ciprofloxacin
Cephalexin
Azithromycin
HOSP RES
UP DOWN
INF PCE
PCE ATE
ATE FCE
FCE DS A
FCE DS B
FCE DOWN
Sites Compared
Significant Differences with Term(s)
Timepoint
Sample Site
Waterway
Waterway and Sample Site
Waterway and Timepoint
Sample Site and Timepoint
Sample Site Waterway and Timepoint

Figure S8

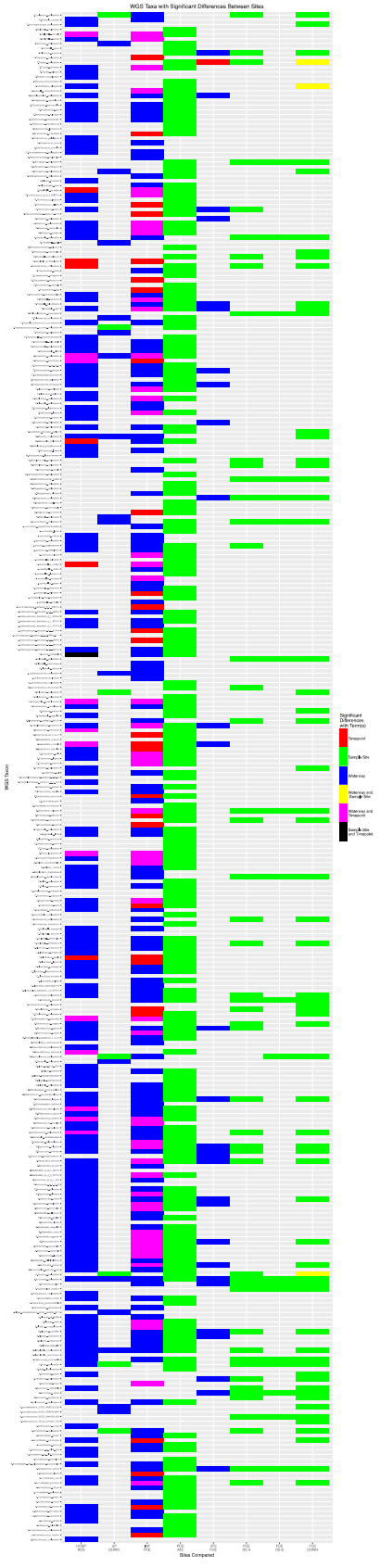
WGS Taxa with Significant Differences Between Sites
WGS Taxon
Significant Differences Seen Term(s)
Timepoint
Sample Site
Waterway
Waterway and Sample Site
Waterway and Timepoint
Sample Site and Timepoint
Sites Compared

Figure S9


ShortBRED Genes with Significant Differences Between Sites
Gene
tetX
tetW
tetQ
tetO
tetE
tetC
tet40
tet39
TEM−93
sul2
sul1
robA
QnrS2
QnrB41
qacH
PmrF
OXA−96
OXA−3
OXA−211
OXA−147
OXA−129
mtrA
msrE
Mrx
mphG
MOX−6
mexT
mel
mefC
mefB
mefA
mdtN
mdtG
marA
lnuC
imiS
H−NS
GES−9
gadW
gadE
FOX−4
evgS
ErmO
ErmG
ErmF
ErmB
EreA2
emrR
emrK
emrB
dfrF
dfrA14
CTX−M−8
CRP
cpxA
cmlA4
CfxA6
CblA−1
catS
cat
baeR
arnA
APH(6)−Id
APH(3")−Ib
APH(3')−IIa
ANT(6)−Ib
ANT(3")−Ii−AAC(6')−IId_fusion_protein
ANT(2")−Ia
aminocoumarin_resistant_cysB
aminocoumarin_resistant_alaS
AER−1
ACT−1
acrF
acrA
aadA15
aadA13
aadA
AAC(3)−VIIa
HOSP RES
UP DOWN
INF PCE
PCE ATE
ATE FCE
FCE DS A
FCE DS B
FCE DOWN
Sites Compared
Significant Differences with Term(s)
Timepoint
Sample Site
Waterway
Waterway and Sample Site

Treated water testing procedures and evaluation criteria

It should be recognized that despite the persistence of limited antibiotics and antibiotic resistance genes ultimately being discharged into local surface waters, this in no way undermines the efficacy and efficiency of the subject wastewater treatment facilities. Currently, the North Carolina wastewater treatment system tests for *Salmonella typhi, Vibrio cholerae, Shigella dysenteraie, E. coli O157:H7,* and *Clostridium perfringens.* For reclamation testing, North Carolina applies fecal coliform measurement limits of <14/100 mL of non-potable water, and values of 3/100 mL for *E. coli,* and 5/100 mL for *Coliphages* and *Clostridium perfringes* in potable sources.

Figure Captions

Figure S1. Concentrations of each antibiotic compound for each sampling location averaged across all four timepoints.

Figure S2. Unique and shared OTUs between Mallard and Sugar Creek treatment facilities from whole-genome shotgun sequencing.

Figure S3. Whole-genome OTUs compared between Mallard and Sugar Creek, stream and sewage samples.

Figure S4. Shannon diversity (A) and Bray-Curtis Beta diversity (B) of all WGS sample replicates. Samples have no significant variance between replicates, demonstrating equivalence utilizing the highest depth replicate.

Figure S5. Species-level taxonomic classification of differentially abundant clades between sampling locations.

Figure S6. Abundance values for antibiotic resistance genes from all timepoints across all sample locations as reported by ShortBRED in RPKM, with red indicating highest abundance

and blue indicating no presence. Sample clustering is based on the Euclidian distance between samples, with overall ARG abundance sorted in decreasing order from left to right. Specific gene terms are listed on the x-axis, with sample names and locations on the y-axis.

Figure S7. Significant differences in antibiotic concentrations between sample site, waterway, and timepoint.

Figure S8. Significant differences in taxa from shotgun sequence analysis between sample site, waterway, and timepoint.

Figure S9. Differentially abundant ARGs in collection sites. ARGs differing with regards to time point, waterway, sample site, and combinations of sample site/time point and waterways are shown. Each colored box indicates a significant abundance difference between the corresponding sites shown on the x-axis, and the significant resistance term on the y-axis. Red, green, and blue indicate substantial differences with regards to timepoint, sample sites, and treatment plant respectively, while yellow shows significant differences at both treatment plant and sample site levels.

File S1. Adjustment values for antibiotic detection and quantification limits.

File S2. Complete linear model statistical results from mass spectrometry data.

File S3. Complete linear model statistical results from taxonomic classification.

File S4. Complete statistical results from mass spectrometry linear models.

File S5. Standard curve calculations for commercial antibiotic standards.

Additional Data

Additional illustrated statistical comparisons for MetaPhlAn2, ShortBRED, and for the mass spectrometry data are available in their entirety from FigShare at https://figshare.com/s/3b6b33ed765fb8605ff2, https://figshare.com/s/5db6e984875ac4062a04, and https://figshare.com/s/1137b9a723c3fa7c0c68 respectively. Heatmaps generated for

visualizing bacterial abundance changes are located at

https://figshare.com/s/fc22b1db16f31491652d.

Supporting Information References

1. Price, M. N.; Dehal, P. S.; Arkin, A. P., FastTree 2--approximately maximum-likelihood trees for large alignments. *PloS one* **2010,** *5*, (3), e9490.
2. Wang, Q.; Garrity, G. M.; Tiedje, J. M.; Cole, J. R., Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* **2007,** *73*, (16), 5261-7.
3. McDonald, D.; Price, M. N.; Goodrich, J.; Nawrocki, E. P.; DeSantis, T. Z.; Probst, A.; Andersen, G. L.; Knight, R.; Hugenholtz, P., An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME J* **2012,** *6*, (3), 610-8.
4. Oliveros, J. C., Venny. An interactive tool for comparing lists with Venn's diagrams. **2007-2015**.
5. Paulson, J. N.; Stine, O. C.; Bravo, H. C.; Pop, M., Differential abundance analysis for microbial marker-gene surveys. *Nat. Methods* **2013,** *10*, (12), 1200-2.