1 *Article*

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

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13

14 **Abstract:**   Wastewater treatment plants (WWTPs) are thought to be potential incubators of
15 antibiotic resistance. Persistence of commonly used antibiotics in wastewater may increase the
16 potential for selection of resistance genes transferred between bacterial populations, some of which
17 may pose a threat to human health. In this study, we measured the concentrations of ten antibiotics
18 in wastewater plant influents and effluents, and in surface waters up- and downstream from two
19 Charlotte area treatment facilities. We performed Illumina shotgun sequencing to assay the
20 microbial community and resistome compositions at each site across four time points from late
21 winter to mid-summer of 2016. Antibiotics are present throughout wastewater treatment, and
22 elevated concentrations of multiple antibiotics are maintained in moving stream water downstream
23 of effluent release. While some human gut and activated sludge associated taxa are detectable
24 downstream, these seem to attenuate with distance while the core microbial community of the
25 stream remains fairly consistent. We observe slight suppression of functional pathways in the
26 downstream microbial communities, including amino acid, carbohydrate and nucleic acid
27 metabolism as well as nucleotide and amino acid scavenging. Nearly all antibiotic resistance genes
28 (ARGs) and potentially pathogenic taxa are removed in the treatment process, though a few ARG
29 markers are elevated downstream of effluent release.   Taken together, these results represent
30 baseline measurements which future studies can utilize to help to determine which factors control
31 the movement of antibiotics and resistance genes through aquatic urban ecosystems before, during
32 and after wastewater treatment.

33 **Keywords:** metagenomics; antibiotic resistance; wastewater; environmental ecology
34

35 1. Introduction

36   Urbanization has the potential to affect surface water quality and alter microbial community
37 composition [1-4].   One mechanism by which human activity directly affects surface waters is
38 through the wastewater treatment process, in which human waste is collected, treated, and the
39 residual water eventually released back into surface waterways [3,5,6].   Pharmaceuticals and
40 antimicrobial compounds that are not fully metabolized or that are disposed of improperly make
41 treated wastewater a significant source of pharmaceuticals in surface waters [7].   Significant effects
42 of pharmaceuticals, including metformin, estrogens, and illegal drugs on native flora and fauna have
43 recently been reported as well [8-11].

With the growth of antibiotic resistance as a public health threat, there has been increased interest in the prevalence of antibiotics and associated resistance elements released to the environment, as well as their removal from wastewater systems [12]. Both antivirals [13] and antibiotics [14] have been found in treated effluent waters in recent studies, exposing the native microbial flora to sub-lethal levels of antibiotics, and contributing to selective pressures potentially resulting in the emergence of resistant strains [15,16]. Agricultural runoff from antibiotic-administered livestock is also of concern [17,18], as is the use of reclaimed water in public locations such as water, amusement, and grassy parks [19]. It is hypothesized that any antibiotic resistance genes (ARGs) persisting in reclaimed water may become long-term environmental contaminants, potentially creating hot spots for breeding resistant microbe populations [20]. Further exploration of reclaimed water systems and efforts to reduce the presence of antibiotics and associated resistance factors are described in a recent review [21].

Results from prior studies of treated effluent impacts on surface waters are highly varied. In areas where water treatment infrastructure is sparse, human microbial resistomes have been shown to pass into the environment through mobile genetic elements [22]. Even in nations with state-of-the-art water treatment systems, the outcome of water treatment is highly dependent on the specific technology used and on operating parameters. For example, a 2011 study showed that treated wastewater was a significant source of antibiotic resistance markers in Minnesota's Duluth Harbor [23]. In contrast, a recent study from Denmark suggests that the dissemination of the resistome is fairly limited [24]. A concurrent study demonstrated that aerobic treatment procedures may significantly reduce antibiotic resistance elements present in wastewater processing locations [25].

The potential for human impact on surface water composition and microbiomes in Charlotte, NC is significant as a city of approximately 800,000 people within a larger metropolitan area of 2.3 million. Charlotte has no large body of water within the city itself, but the surrounding Mecklenburg County does have a network of over 3000 miles of small creeks and streams, many of which are integral features of popular public greenway and park facilities. The regional water utility, Charlotte Water, operates 5 major wastewater treatment and 2 minor package plant facilities that release treated wastewater into local creeks. The purpose of this study is to establish a baseline understanding of the impact of treated wastewater release on the urban stream microbiome, and to quantify the impact of released effluent on antibiotic concentrations and resistance elements observed in the streams.

2. Materials and Methods
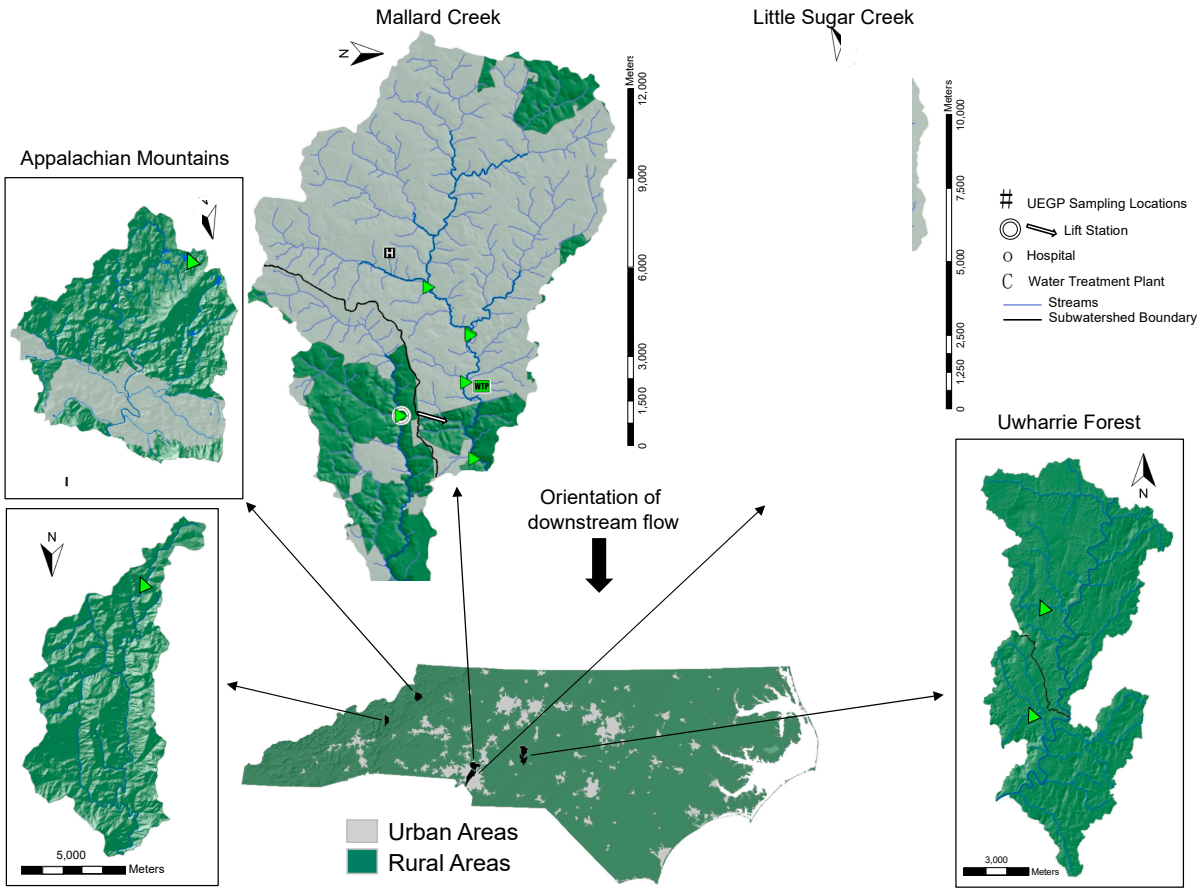
2.1. Selection of sampling sites

The design of this study encompasses two systems: the urban stream upstream and downstream of treated wastewater release points, and the wastewater treatment plant and its various processing stages. Our primary interest was in the impact of treated wastewater release on the stream environment, which has the most obvious consequences for the population of Charlotte that interacts with these streams in public areas, but we also sought insight into the impact of sewage input sources and various stages in the water treatment process on the final released product.

The sites selected for this study, Mallard Creek (MC) and Sugar Creek (SC) reclamation facilities, are activated sludge plants that release treated wastewater into Mallard Creek and Little Sugar Creek. These streams are in two different watersheds; Mallard Creek feeds into the Yadkin Pee-Dee watershed, while Little Sugar Creek feeds into Sugar Creek and ultimately into the Catawba River basin. Sampling sites chosen in the urban streams include two points upstream of treated wastewater release and two points downstream, at distances of approximately 3300 and 2700 meters from effluent release for Little Sugar and Mallard Creek respectively. Two remote locations were also selected in the Appalachian Mountains (MNTA/MNTB) and Uwharrie forest preserve (UWHA/UWHB) to give insight into the microbial community and antibiotic load at sites more removed from urban activities. While these sites differ in their elevation and therefore in their

95   underlying geology, they were our best option for a remote background due to the lack of Piedmont
96   stream sites that are relatively untouched by human activity.
97   We sampled several sites inside each WWTP, representing processing stages.   Five sites were
98   selected inside the Mallard Creek plant, including the raw influent (INF), the primary clarifier
99   influent (PCI), the primary clarifier effluent (PCE), the aeration tank effluent (ATE), and the final
100   clarifier effluent (FCE).   Samples from both hospital-adjacent (HOSP) and residential (RES) sewage
101   trunklines "upstream" of the plant were collected in each case, to allow investigation of potential
102   microbial community differences between residential and hospital waste. Corresponding locations
103   were used at the Sugar Creek plant, with sites including INF, PCE, ATE, and FCE.   Location of
104   sampling points at the Sugar Creek plant did not allow for collection of a PCI sample, but it was
105   possible to directly sample ultraviolet disinfected effluent (UV) which had not been accessible at the
106   Mallard Creek plant.
107   The locations of the plants and sampling sites are illustrated in Figure 2, with full details of
108   sample collection and handling provided in SI.
109

110
111   **Figure 1.**   Geographical illustration of sampling sites and locations.   Mountain samples (A,B) were
112   collected in Caldwell and Yancey counties respectively, while both Uwharrie forest samples were
113   collected in Stanly county.   Mallard and Sugar Creek facilities and trunklines were all located in
114   Mecklenburg county.   Urban locations are marked in grey, while more rural locations are shaded
115   green.   Hospital and wastewater treatment plant locations are indicated in relation to sample
116   collection sites, which are denoted with green triangles.
117

2.2. Treatment methods and conditions in Charlotte Water facilities

119
120   Both plants studied use similar activated sludge treatment processes, beginning with physical
121   bar screens, grit removal stations, and primary clarifiers, followed by activated sludge processing
122   consisting of anoxic and aerobic zones and secondary clarifier tanks, and ending with ultraviolet

123   disinfection at an intensity of 16,000 µW-sec/cm². The Sugar Creek facility employs an additional
124   filtration step with anthracite bed filters, followed by wet and dry odor scrubber units, prior to creek
125   release. Anaerobic digestion is used for solids treatment with digestate returned to the activated
126   sludge tank, and biosolids reclamation is conducted at both facilities. An overview of this process is
127   shown in Figure 1. Final effluent is monitored for carbonaceous biochemical oxygen demand (CBOD),
128   total suspended solids (TSS), ammonia, chronic toxicity (*Ceriodaphnia dubia*), fecal coliform, flow rates,
129   dissolved oxygen, pH, and phosphorus. Sugar Creek also monitors nickel and copper
130   concentrations. The Mallard Creek facility is rated for 12 million gallons daily (MGD) released
131   directly into Mallard Creek, while the Sugar Creek facility is rated for 20 MGD and discharges into
132   Little Sugar Creek.
133
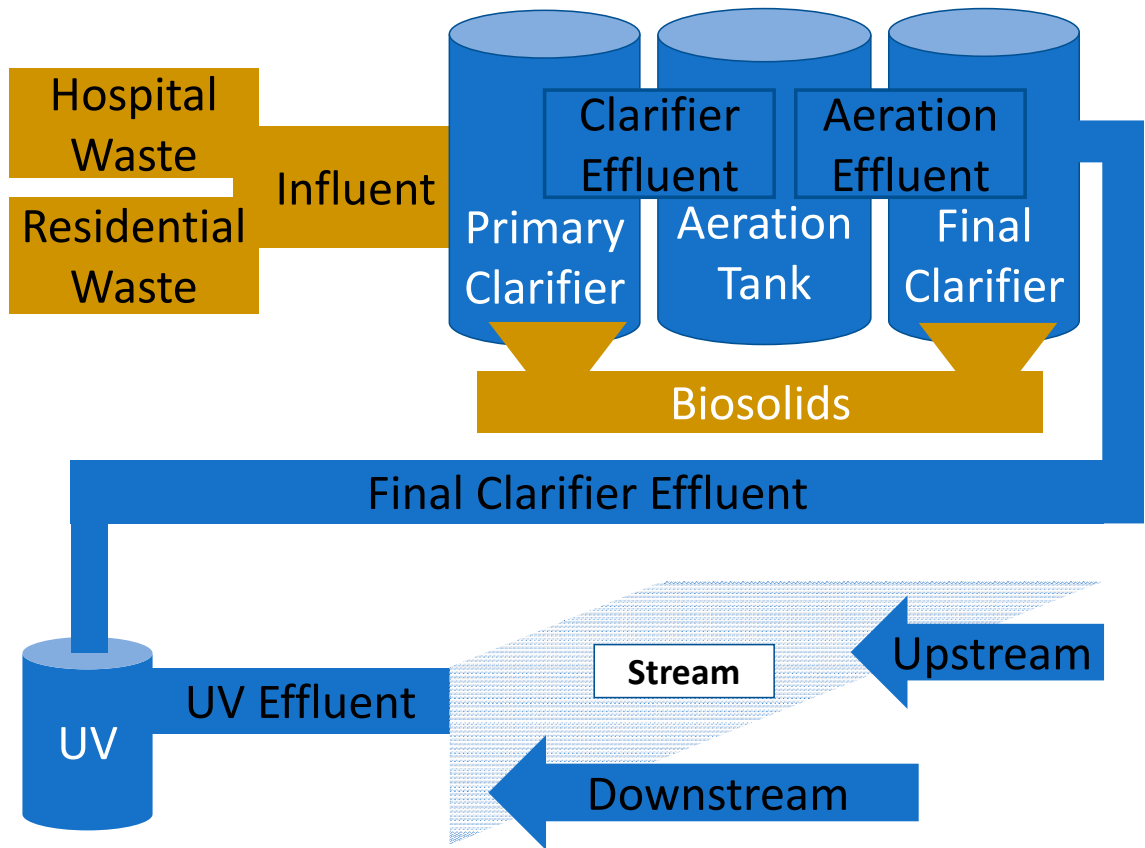
135   **Figure 2.** Summary of the wastewater treatment process in Charlotte facilities. Untreated
136   wastewater from trunkline locations, including hospital and residential wastes, collects into the main
137   influent trunkline before entry into the treatment plant. The primary clarification tank is the first
138   stage in treatment, removing physical debris and solids. The aeration tank assists in removing oils
139   and hydrocarbons before a final physical precipitation in the final clarifier. Solids are collected in both
140   the primary and final clarifier tanks for reuse. Effluent from the final clarifier is sterilized in an
141   ultraviolet treatment tank before discharge into environmental streams.
142

143   *2.3. Sample Collection and Handling*
144

145   Stream samples were collected by submerging sterile 1L Nalgene screw cap bottles
146   approximately 6 inches beneath the water surface until full. The bottle mouth was oriented against
147   the direction of flow to prevent any disturbed upstream sediment from being received and was
148   capped while submerged. A total of 6 L of creek water was collected from each creek sampling site
149   and stored in an insulated cooler for transport. Sewage samples were collected using an ISCO 6712
150   auto-sampler, pulling 150 mL of sewage every 30 minutes peristaltically to generate a 24-hour
151   composite volume in sterile 2.5 gallon carboys. The carboys were kept in an ice water bath within
152   the sampler during the collection time. Composite collections at the reclamation facilities also used

153 the same sampling strategy with refrigerated ISCO 6712FR autosamplers and carboy storage at 4
154 degrees Celsius for 24 hours.   Following collection, 1 L of volume was transferred via a peristaltic
155 pump from the collection carboys after thorough mixing into 1 L sterile Nalgene bottles in a sterile
156 hood for further processing.   An additional 500 mL was pumped into sterile amber glass bottles
157 containing 200 mg of EDTA to inhibit bacterial growth for mass spectrometry analysis of antibiotic
158 residues.   In total, four time points were collected beginning with late Winter (time point 1), early
159 and late Spring (time points 2 and 3 respectively), and mid-Summer (time point 4).   The pump
160 tubing was replaced between each sample collection and the bottles were stored at 4 degrees Celsius
161 until DNA extraction.   At the time of sample retrieval, environmental metadata measurements were
162 collected along with the sample material.   These data were integrated in downstream correlation
163 analyses and models.   Metadata gathered at the time of collection included dates and times, latitude
164 and longitude, ambient, sample, and storage temperatures, conductivity (pHmv), pH, humidity, and
165 autosampler composite collection start and end times (if applicable).
166
167 *2.4. Detection of antibiotic compounds by mass spectrometry*
168
169   Ten antibiotic compounds representing a broad range of commonly prescribed classes were
170 chosen for the study.   These included sulfamethoxazole, trimethoprim, ciprofloxacin, cephalexin,
171 levofloxacin, amoxicillin, clindamycin, doxycycline, ertapenem (used exclusively for multi-drug
172 resistant infections in hospitals), and azithromycin.   Standardized compounds were used to
173 generate calibration curves for detection of each antibiotic in the wastewater composites, treated
174 wastewater, and stream collections.
175   Antibiotic standards for each were spiked into various solvents, according to their preparation
176 instructions.   Ciprofloxacin, doxycycline, ertapenem and amoxicillin were spiked into sterile water
177 in varying concentrations from 1.95 ng/mL to 1000 ng/mL.   Azithromycin, clindamycin, and
178 sulfamethoxazole were spiked into ethanol, and levofloxacin, trimethoprim, and cephalexin were
179 spiked into methanol at the same concentrations.   An additional curve was generated for amoxicillin
180 at diluted concentrations from 0.12 ng/mL to 0.98 ng/mL.   Ciprofloxacin required an additional
181 curve, as the initial data points were not linear.   500 mL composites of previously described
182 wastewater and creek samples were passed over Whatman microfiber and 0.47 micron membrane
183 filters (GE healthcare) prior to cartridge loading.   EDTA was added before and after filtering to
184 inhibit bacterial growth and to chelate any metal ions.   Each sample was adjusted to a pH of 3.5 with
185 10% formic acid, and 200 mL of each sample were loaded onto preconditioned Oasis HLB cartridges.
186 Each cartridge was washed with 12 mL of sterile water and 12 mL of methanol and formic acid
187 (99%/1% v/v) to elute the bound material, which was subsequently dried overnight in nitrogen.
188 Following desiccation, 190 uL of 99%/1% methanol/formic acid with 10 uL of 200 ng/mL Cl-
189 phenylalanine was added to the dried material.   Samples were vortexed for 10 minutes and
190 centrifuged at 10,000xg for 5 minutes before the supernatant was transferred to vials for UPLC-MS
191 analysis.   In total, 26 samples were loaded into an Acquity UPLC-Quattro Premier XE MS system
192 (Waters Corp, Milford, MA) and ran in positive electrospray ionization mode.   Raw files were
193 processed using the TargetLynx Application Manager (Waters Corp, Milford, MA), obtaining values
194 of peak area and retention time.   Concentrations of antibiotic compounds from each sample were
195 calculated from the spray spectra based on the standard antibiotic curves.   A total of 92 samples
196 were processed in four batches so that preparation of the samples and the UPLC-MS analysis could
197 be conducted on the same day.   Antibiotic standard dilutions were also run in series with each batch,
198 along with an internal phenylalanine standard to ensure consistency in instrument performance.
199
200 *2.5. DNA Extraction*
201
202   All samples were passed in triplicate over 0.45 micron vacuum water filters (Mobio) in 100 mL
203 volumes until the flow had stopped.   Flow-through volumes varied from ~150 mL to ~1 L depending
204 on the turbidity and origin of the sample.   Filters were removed from the filtration unit with

205   sterilized forceps and halved with sterile scissors, where one half was sliced into ~5mm wide strips
206   and the other was stored at -80 Celsius for future redundancy.   The filter material strips were then
207   placed in a bead homogenizer tube of the FastDNA SPIN kit for soil (MP Biomedicals) and
208   homogenized for 60 seconds using a benchtop FastPrep-24 homogenizer (MP Biomedicals) as
209   recommended in the manufacturer's manual.   DNA extraction and elution were conducted
210   according to the manufacturer's protocol for increased yield and quantified using a Qubit
211   fluorometer (Thermo-Fisher) and Nanodrop spectrophotometer (Thermo-Fisher).   Following DNA
212   quality control, samples were frozen at -20 Celsius before delivery to the David H. Murdock Research
213   Institute (DHMRI) Genomics laboratory for sequencing library preparation.   Primer, adapter, and
214   barcode sequences are listed in the SI.
215
216   *2.6. Shotgun metagenomic library preparation and sequencing*
217
218   All sequencing was performed by the core laboratory at the David H. Murdock Research
219   Institute (DHMRI).   Amplicon libraries were generated from collected DNA templates and
220   validated using qPCR.   Each was uniquely indexed and all samples were pooled together in
221   equimolar proportions and sequenced with 125bp paired-end reads on an Illumina HiSeq 2500 flow
222   cell.   Time points 1-3 each comprised 66 total samples, while time point 4 consisted of 78 total
223   samples including the Uwharrie and Appalachian locations.   Sequences were demultiplexed and
224   debarcoded by the DHMRI team prior to delivery.   Libraries were pooled nine per HiSeq 2500 lane,
225   resulting in a sequencing depth of ~5Gb per sample with 125bp paired-end reads.
226
227   *2.7. DNA Sequence Trimming and Quality Control*
228
229   Raw DNA sequences were filtered and demultiplexed by the DHMRI using the Illumina HiSeq
230   HCS software (Illumina).   Greater than 80% of the bases must have a quality score greater than 30,
231   or an accuracy rate of 99.9%.   Barcode, primer, and adapter sequences were also removed and
232   verified in-house before proceeding with further analysis.   Trimmomatic parameters for read
233   clipping were ILLUMINACLIP:TruSeq3-PE:2:30:10 to ensure complete removal of any remaining
234   Illumina adapters.   Leading and trailing base calls below a PHRED score of 3 were removed, and a
235   sliding window approach was implemented with a required average PHRED score of 20 across a 3-
236   base section.   Lastly, a minimum read length of 50 bases was specified, retaining ~80% of the total
237   input reads, which were merged using PEAR with a minimum specified overlap of 10 bases,
238   minimum assembled length of 50 bases, and a minimum alignment p-value of 0.01, resulting in an
239   average assembly efficiency of 99%.   1,344,546,211 paired reads for each deep-sequenced sample
240   were obtained and used to ensure the multiplexing strategy did not affect or dilute the results
241   returned for pooled samples.   For the pooled metagenome shotgun sequences, approximately 88%
242   of the total reads per sample contained surviving paired mates following quality control, yielding an
243   average of 147,163,056 reads for each multiplexed sample.
244
245   *2.8. Metagenomic classification analysis using MetaPhlAn2*
246
247   To determine species-level relative abundance, we analyzed the merged shotgun sequence
248   datasets with the Metagenomic Phylogenetic Analysis for Metagenomic Taxonomic Profiling
249   (MetaPhlAn) package, version 2.5.0 [26].   Sequences were aligned to the default MetaPhlAn2 marker
250   database (v.20) for relative abundance measurements.   Heat maps of the top 100 species-level taxa
251   were generated in R [27] with ggplot2 [28], using hclust and Bray-Curtis distance calculations, along
252   with alpha and beta diversity metrics (links provided in SI).   Bar plots were generated using ggplot2.
253

2.9. Identification and quantitation of resistance elements using ShortBRED

Antibiotic resistant markers were identified and quantified using Short, Better Representative Extract Data (ShortBRED) [29]. To ensure the broadest available reference database for alignments, we created a custom database with ShortBRED-identify, consisting of the Comprehensive Antibiotic Resistance Database (CARD) [30] version 1.1.0 (August 2016) merged with the Lahey Clinic beta-lactamase database. ShortBRED-quantify was applied to the merged forward and reverse paired shotgun sequencing reads. We used the highest depth replicate to calculate the representative normalized reads per kilobase per million mapped reads (RPKM) counts of hits to each marker in the database for each sample. All markers with zero RPKM hits were then removed. Heat maps were generated from the top 40 markers with the highest number of normalized counts and barplots were created to show overall ARG load at each sampling location.

2.10. Functional classification analysis using HUMAnN2

To determine pathway-level relative abundance, we analyzed the merged shotgun sequencing datasets with the HMP Unified Metabolic Analysis Network (HuMAnN2) package, version V0.11.1. Sequences were aligned to the ChocoPhlAn nucleotide database and a translated search was performed using the Uniprot UniRef90 [31] database. Pathway abundance files generated by HuMAnN2 were used for downstream statistical analysis.

2.11. Statistical Methods

The significance of differences in microbial relative abundances, pathway relative abundances, and ShortBRED-derived resistance elements between sites were each assessed using linear regression models, via the lm function in R [27]. The relative abundance or RPKM of ShortBRED genes served as the response variable and the final explanatory variables consisted of the stream source (MC or SC), the sampling site (upstream, treatment plant influent, downstream, etc.), and a representation of the time at which the sample was taken (late winter, early spring, late spring, mid-summer). As the triplicates were clustered closely together, the most deeply sequenced sample from each set of replicates was taken as the representative for that measurement. A threshold frequency of a non-zero presence in at least 25% of all samples in the comparison was used to avoid wasting hypotheses on stochastic differences in rare taxa or genes. The Benjamini-Hochberg false discovery rate was used to perform multiple hypothesis correction [32]. All p-values are Benjamini-Hochberg corrected, and complete statistical comparison results are available at the links provided in SI.

3. Results

Our study of antibiotics, taxa and resistance genes throughout the Charlotte, NC urban watershed surveyed several processing stages inside two wastewater treatment plants, as well as upstream and downstream sites across four time points in the 2016 calendar year. We found that the release of treated water maintains elevated concentrations of multiple antibiotics in downstream waters, and that some pathogens of interest are present in small quantities in the streams, both upstream and downstream of treatment sites. However, unlike antibiotic compounds, pathogenic taxa and antibiotic resistance gene markers are generally not significantly increased in concentration in moving waters downstream of treated water release.

3.1. Antibiotic concentrations are elevated downstream of wastewater treatment plants

Ten compounds were chosen to represent a diversity of frequently used classes of antibiotics with different chemical properties and mechanisms of action. Eight of these (ciprofloxacin, doxycycline, azithromycin, clindamycin, sulfamethoxazole, cephalexin, trimethoprim, and

305   levofloxacin) were detected in most of the samples collected (supplemental figure S1).   By contrast,
306   ertapenem and amoxicillin were only seen in a limited number of samples at concentrations below
307   the limit of quantification (1.95 and 0.24 ng/L respectively).
308   Our survey included two urban upstream sites (Mallard and Little Sugar Creek) as well as two
309   rural sites chosen for low human impact, which we anticipated would have low background
310   antibiotic concentrations.   Remote rural sites and upstream sites were substantially equivalent, with
311   antibiotic concentrations changing by an average of less than 5 ng/L when compared to each other,
312   with no significant differences with regards to the presence of specific antibiotics, and no significant
313   seasonal changes ($p<0.05$, Supporting File S2).   Consistent with previous literature [14,33,34],
314   concentrations of the eight consistently detected antibiotics were present in measurable
315   concentrations throughout the treatment process (Figure 3).   For both Little Sugar and Mallard
316   Creek locations, downstream concentrations of all antibiotic compounds were significantly increased
317   relative to their upstream concentrations, by an average factor of 8-fold, except for clindamycin
318   (Figure S3).   This demonstrates whole-compound persistence throughout the treatment plants and
319   into the discharged treated wastewater.   We also compared two downstream sites at distances of
320   3300 and 2700 meters for both Little Sugar and Mallard Creek respectively and found that overall
321   antibiotic concentrations decreased significantly by an average of 6 ng/L from the more proximal
322   downstream site A to the more distal downstream B site (Figure 3), with the largest concentration
323   reduction seen in ciprofloxacin.   Overall, these data demonstrate that wastewater treatment plant
324   effluents can act a source of antibiotics, sufficient to maintain elevated concentrations of antibiotics
325   in moving water for a considerable distance downstream of the plant.
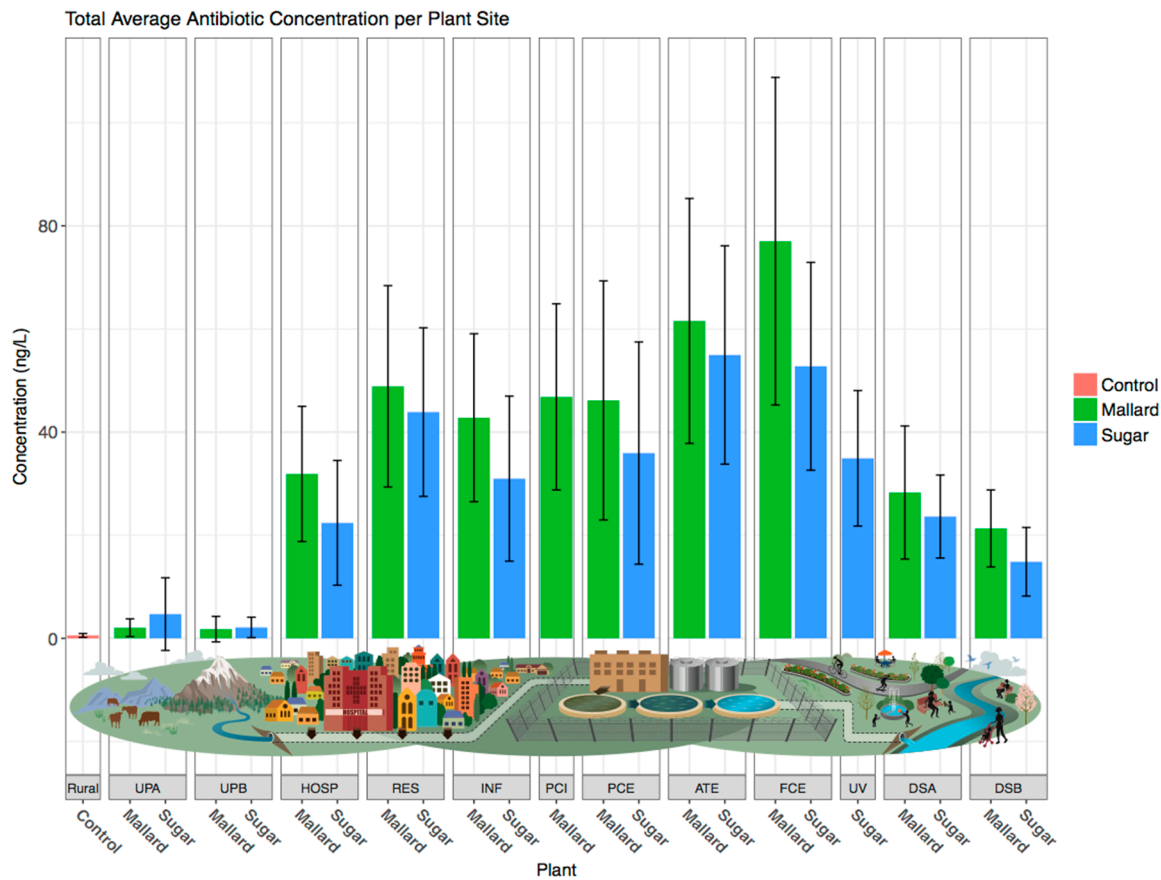
326
327   **Figure 3.**   Antibiotic concentrations for each sampling site.   Concentrations for all ten antibiotic
328   compounds, reported in ng/L, were averaged across all four time points for each sampling location and
329   treatment plant.   Standard deviations are reported within each concentration bar.   Rural sites are
330   shown in red for comparison to urban wastewater, with Mallard and Little Sugar Creek sites in green
331   and blue colors respectively.   PCI is reported for Mallard Creek only, and UV is only reported for the
332   Sugar Creek facility, as described in Methods.

3.2. Treated wastewater microbial communities become more like fresh water communities as waste progresses through the treatment process

Relative abundances of microbial species were computed from shotgun sequencing data using MetaPhlan2 [26] (Figure 4). Influent samples and sewage prior to the ATE stage were the richest in species diversity, while stream samples and treated wastewater were the least diverse (Figure 5). Beta-diversity measures showed that treated water samples clustered together, separately from stream and rural sites (Figure 6). Technical replicates conducted in triplicate with independent sample DNA extraction and sequencing displayed little variance (Figure S4).

When we examine upstream and downstream stream samples at the phylum level Actinobacteria and Proteobacteria predominate (Fig. 4), as is typical for freshwater communities [35]. At the genus level, 366 total taxa were detected, 16 of which were significantly different between upstream and downstream locations. Of these, 15 were higher in relative abundance downstream, and are also present in at least 2 wastewater treatment plant locations at a relative abundance of greater than 0.1% (Table S1). These results are consistent with these taxa being introduced into the downstream ecosystem from the wastewater treatment plant. However, when we compared the closest (DSA) and most distant (DSB) downstream locations from the plant effluent discharge sites to see whether any of these species decrease in relative abundance with increased distance from the effluent sources we found no significant differences in enriched taxa with respect to distance from the discharge site (data not shown).

Within the influents and the treatment plant environment, Bacteroides and Firmicutes predominate (Fig. 4), as we would expect from previous literature [36,37] for material mainly originating in the human gut. Collectively, raw sewage influents between the Mallard and Sugar Creek treatment facilities were comparable, with no significant population deviations between the two basins at the phylum level ($p<0.05$). Minimal differences in microbial relative abundance were detected between mixed hospital waste and waste that was exclusively residential, with the soil dwelling bacterium *Kocuria rhizophila* (p=0.0318) as the only significantly different taxon (Table 1). When influents pass through the aeration tank following the point at which activated sludge is introduced, there is a noticeable shift in dominant phyla with 23 taxa being significantly different (Table 1). *Actinobacteria* species are reintroduced, and the trending relative abundances of Bacteroides and Firmicutes drop, although those phyla are still more abundant than they are in the stream. When upstream locations and rural sites were compared, the soil-associated genus *Sphingobium* was the only significant differing taxon (p=0.01) detected at elevated levels in the rural sites when compared to Charlotte upstream locations (Table 1).

3.3. Microbial community shifts during wastewater processing

Comparisons between wastewater collection and treatment stages were constrained to processing stages that were identical between the two plants. No significant differences in taxa were observed between the PCI and PCE stages where there is no active processing. However, between the PCE and the aeration tank effluent (ATE), 221 unique taxa were significantly altered in relative abundance with 192 of these being reduced overall. Of the taxa that increased in relative abundance in the aeration tank effluent, all have been previously characterized as digestors and denitrifiers within wastewater and/or activated sludge [36,38-55].

In the subsequent treatment stage from the ATE to the final clarifier effluent (FCE), we also observed very minor changes in relative abundance, although several differences were marginally significant (Supporting File S3). The composition of the FCE prior to ultraviolet disinfection in both plants differed significantly from downstream samples across 66 different taxa. Most of the differential taxa appeared to be reduced from FCE levels during the subsequent UV disinfection step (Table 1)

384   The internal population profiles of both Mallard and Sugar Creek plants were generally
385   quite similar.   At both the late winter (Fig. 4) and the other collection timepoints (Figure S4), the FCE
386   of Sugar Creek had significantly lower relative abundances of the soil- and   freshwater- associated
387   genus *Thauera,* when compared to Mallard Creek FCE.   All comparisons between plant stages can
388   be accessed in their entirety from the Figshare links provided in the Supplemental Information.
389
390   **Table 1.**   Taxa of significant differential abundance between wastewater treatment stages and stream
391   sites.   The sampling location containing a higher percent abundance of the listed taxa is shown in the
392   "Higher Abundance" column, and the Bonferroni-corrected *p* value resulting from the mixed linear
393   models is also shown**.**

| Taxa	*p* Value	Higher Abundance
Peptostreptococcaceae	0.0039	Downstream to Upstream
Afipia	0.0093	Downstream to Upstream
Holospora	0.0039	Downstream to Upstream
Azoarcus	0.0114	Downstream to Upstream
Acinetobacter	0.013	Downstream to Upstream
Bppunalikevirus	0.0093	Downstream to Upstream
Yualikevirus	0.0096	Downstream to Upstream
Sphingobium	0.01	Rural to Upstream
Kocuria rhizophila	0.0318	Residential to Hospital
Nitrospira defluvii	0.0216	ATE to PCI
Caulobacter sp	0.0058	ATE to PCI
Afipia clevelandensis	0.0048	ATE to PCI
Rhodopseudomonas paulustris	0.012	ATE to PCI
Hyphomicrobium denitrificans	0.0114	ATE to PCI
Mesorhizobium sp	0.0183	ATE to PCI
Paracoccus sp	0.0439	ATE to PCI
Reyranella massiliensis	0.0111	ATE to PCI
Sphingobium xenophagum	0.0184	ATE to PCI
Sphingopyxis sp	0.0003	ATE to PCI
Alicycliphilus sp	0.0004	ATE to PCI
Limnohabitans sp	0.0005	ATE to PCI
Polaromonas sp	0.0003	ATE to PCI
Variovorax sp	0.0014	ATE to PCI
Azoarcus sp	0.0006	ATE to PCI
Dechloromonas sp	0.011	ATE to PCI
Methyloversatilis sp	0.0008	ATE to PCI
Thauera aminoaromatica	0.0212	ATE to PCI
Actinobacter parvas	0.025	ATE to PCI
Turneriella parva	0.0058	ATE to PCI
Methanobrevibacter sp	0.0357	ATE to PCI
Gordonia amarae	0.0476	ATE to PCI
Tetrasphera elongata	0.0218	ATE to PCI
Rhodococcus	0.0409	Downstream to FCE
Actinobacterium sp	0.0116	Downstream to FCE
Polynucleobacter necessarius	0.00000007	Downstream to FCE
Limnohabitans	0.00000007	Downstream to FCE
Methylotenera	0.0404	Downstream to FCE
Bppunalikevirus	0.0132	Downstream to FCE
Yualikevirus	0.0266	Downstream to FCE

394
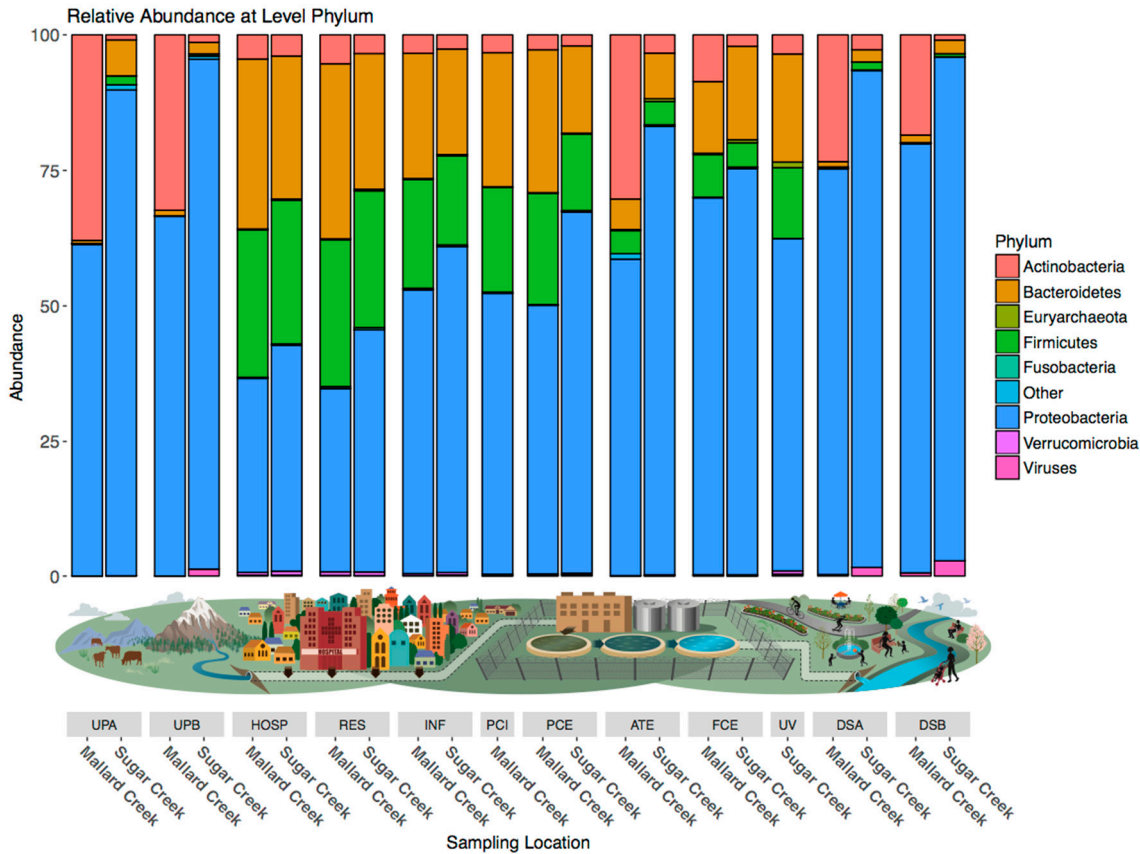
Figure 4. Relative abundance of phyla in collection sites for shotgun sequence data.   Abundance
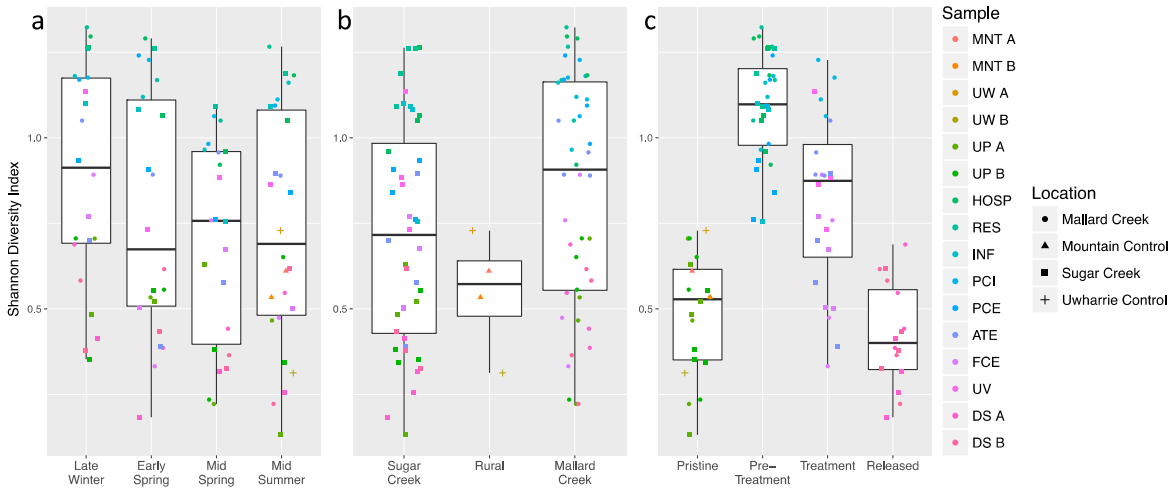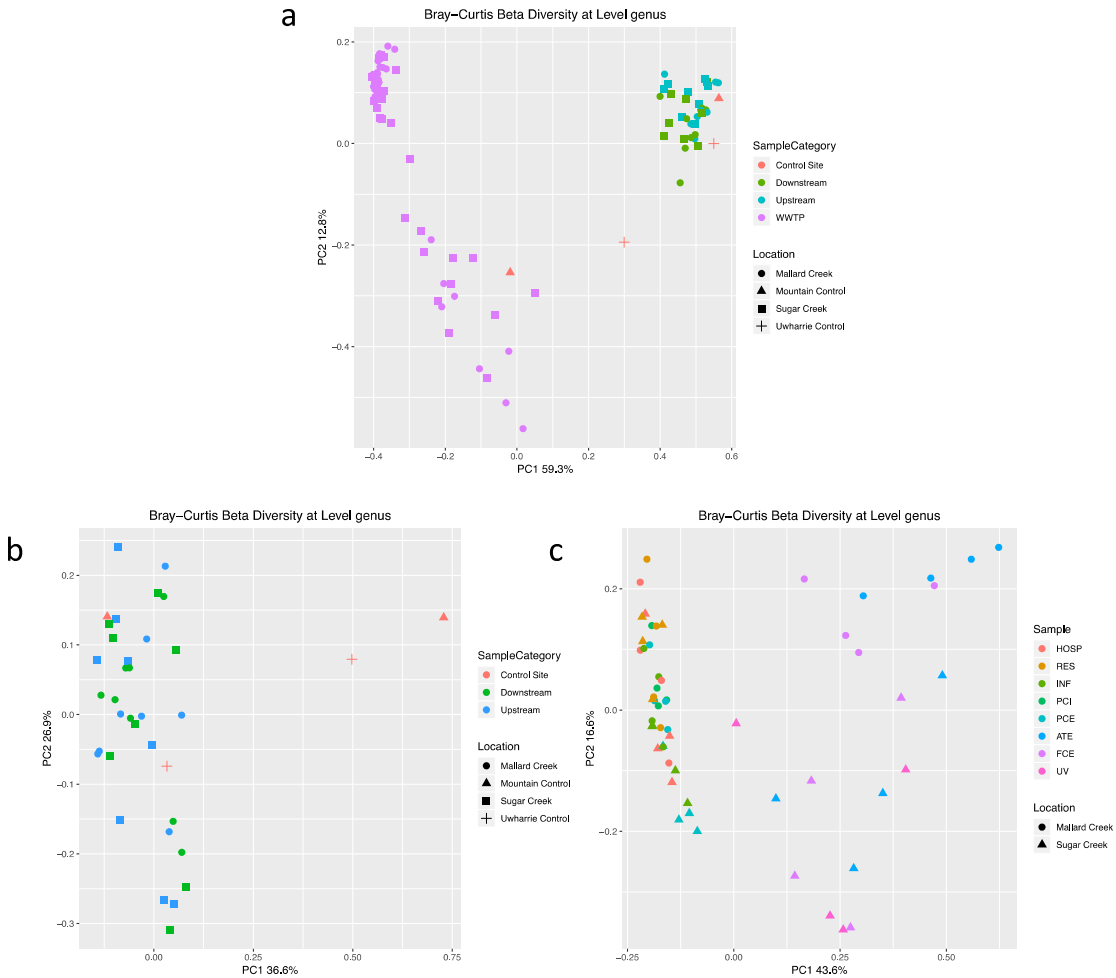
wastewater samples (a), stream samples only (b), and wastewater samples only (c).   Raw, unprocessed wastewater samples cluster together, while streams and processed wastewater (ATE+) have similar clustering patterns.   Rural locations cluster with background urban streams, and the greatest separation between samples appears to be driven most by treatment type.

3.4. Shifts in stream microbial community function are observed downstream of treated wastewater release points

In order to understand the potential functional significance of observed changes in the microbial communities among the locations sampled, we analyzed the data using HUMAnN [56] to determine the relative abundance of orthologous gene families making up known MetaCyc microbial pathways [57]. Between upstream and downstream sites, there are 24 significant pathway relative   abundance changes ($p<0.05$) (Table 2).   Twenty-two of the 24 affected pathways are present in higher relative abundance upstream of treated water release.   Pathways involved include nucleotide, amino acid and carbohydrate biosynthesis, as well as nucleotide and peptide degradation and salvage pathways.

Table 2. Metabolic pathways significantly enriched in up or downstream locations.   Significant MetaCyc functional pathways from HUMAnN are shown, along with their corresponding *p* values and whether they were enriched upstream or downstream of treatment plant effluent release.

| MetaCyc Pathway	p-value	Highest Abundance
PWY.5747..2.methylcitrate.cycle.II	0.009	Upstream

|		
PWY.5659..GDP.mannose.biosynthesis	0.010	Upstream
PWY0.42..2.methylcitrate.cycle.I	0.010	Upstream
GLYCOGENSYNTH.PWY..glycogen.biosynthesis.I..from.ADP.D.Glucose.	0.042	Upstream
ILEUSYN.PWY..L.isoleucine.biosynthesis.I..from.threonine.	0.042	Upstream
PWY.5109..2.methylbutanoate.biosynthesis	0.042	Downstream
PWY.5973..cis.vaccenate.biosynthesis	0.042	Upstream
PWY.6606..guanosine.nucleotides.degradation.II	0.042	Upstream
PWY.6609..adenine.and.adenosine.salvage.III	0.042	Upstream
PWY.7111..pyruvate.fermentation.to.isobutanol..engineered.	0.042	Upstream
PWY.7198..pyrimidine.deoxyribonucleotides.de.novo.biosynthesis.IV	0.042	Upstream
PWY.7199..pyrimidine.deoxyribonucleosides.salvage	0.042	Upstream
PWY.7208..superpathway.of.pyrimidine.nucleobases.salvage	0.042	Upstream
PWY.7210..pyrimidine.deoxyribonucleotides.biosynthesis.from.CTP	0.042	Upstream
PWY.7211..superpathway.of.pyrimidine.deoxyribonucleotides.de.novo.biosynthesis	0.042	Upstream
PWY.7663..gondoate.biosynthesis..anaerobic.	0.042	Upstream
SALVADEHYPOX.PWY..adenosine.nucleotides.degradation.II	0.042	Upstream
UNINTEGRATED	0.042	Upstream
UNMAPPED	0.042	Downstream
VALSYN.PWY..L.valine.biosynthesis	0.042	Upstream
PWY.3781..aerobic.respiration.I..cytochrome.c.	0.042	Upstream
PWY0.1261..anhydromuropeptides.recycling	0.042	Upstream
GLUTORN.PWY..L.ornithine.biosynthesis	0.045	Upstream
PWY.6608..guanosine.nucleotides.degradation.III	0.045	Upstream

429

430   *3.5. Resistance genes are slightly more abundant downstream of wastewater treatment plants*

431

432   In order to measure changes in resistance gene content, we used ShortBRED to identify specific
433   antibiotic resistance associated genes and elements.   Alignment of shotgun sequencing data to the
434   hybrid CARD and Lahey databases for antibiotic resistance associated genes and mobile plasmid
435   elements revealed hits to a total of 600 unique terms from all of the samples across all time points.
436   Of the 600 total detected antibiotic resistance associated sequences, 9 were more abundant in
437   downstream waters when compared to upstream (statistical tables available from FigShare links in
438   SI).   These included carbenicillin and oxacillin beta-lactamases CARB-3 and OXA-1 from the WHO
439   priority pathogen *Pseudomonas aeruginosa*, including two extended spectrum beta-lactamases (Figure
440   7B and Figure S6).   Plasmid-derived sulfonamide resistance for *Vibrio cholerae* species, encoding a
441   dyhydropteroate synthase, was also more abundant in downstream waters.   Multiple genes
442   conferring multi-drug resistance (MDR) within the *E. coli* K-12 strain were detected as well, all
443   encoding multiple efflux pump subunits or modulating efflux control.   Overall, only two antibiotic
444   resistance elements were significantly different in relative abundance between upstream and
445   downstream sites.   A *Streptomyces lividans* methyltransferase was found in significantly higher

446   abundance upstream (*p*=0.044), while a beta-lactamase from *Pseudomonas aeruginosa* was higher
447   downstream (*p*=0.044).
448   When compared to relative abundances of ARG sequences within the FCE stage of treatment, 15
449   of the 600 terms were significantly lower downstream (statistical tables provided in FigShare).   An
450   *Exiguobacterium* macrolide efflux pump (*p*=0.043) was detected in higher relative abundance within
451   partial hospital sewage, while the residential sewage contained significantly more *Escherichia coli*
452   macrolide efflux pump and associated MDR efflux pump membrane proteins (*p*=0.043). Following
453   treatment in the primary clarifier, no ARGs were altered significantly in abundance from the raw
454   influent.   Subsequent digestion in the aeration tank reduced the relative abundance of 84 antibiotic
455   resistance elements, with many falling to undetectable levels.   The transition from ATE to FCE did
456   not result in further significant changes to relative abundance of any ARG markers, although a
457   methyltransferase from *Streptomyces lividans* and an aminoglycoside acetyltransferase in *Streptomyces*
458   *rimosus* were marginally reduced (*p*=0.052 and 0.051 respectively).   The final UV step (implicit in the
459   comparison of FCE to downstream sites DSA and DSB) resulted in the reduction or removal of twelve
460   additional markers, including a *Vibrio cholerae* and *Vibrio fluvialis* sulfonamide dihydropteroate
461   synthase (*p*=0.0004), an aminoglycoside acetyltransferase in *Serratia marcescens* (*p*=0.014), a
462   *Pseudomonas aeruginosa* beta-lactamase (*p*=0.054), a tetracycline efflux pump in *Acinetobacter* (*p*=0.054),
463   *Photobacterium damselae* macrolide efflux pump (*p*=0.054), *Escherichia coli* plasmid-encoded efflux
464   pumps for streptogramin, streptomycin, and erythromycin (*p*=0.054), and suggest elevated levels of
465   a chloramphenicol acetyltransferase in *Morganella morganii,* and an MDR efflux pump for
466   *Mycobacterium tuberculosis* (*p*=0.1).   No significant differences in ARG markers were detected
467   between the two downstream locations.   Remote mountain and Uwharrie stream sites and the
468   upstream urban sites were essentially equivalent, with no significant difference in antibiotic
469   resistance markers.   Corresponding illustrations for individual antibiotic concentrations and
470   shotgun analysis, ARGs and all taxa, as well as between site comparisons are provided in SI (Figures
471   S7-S9).
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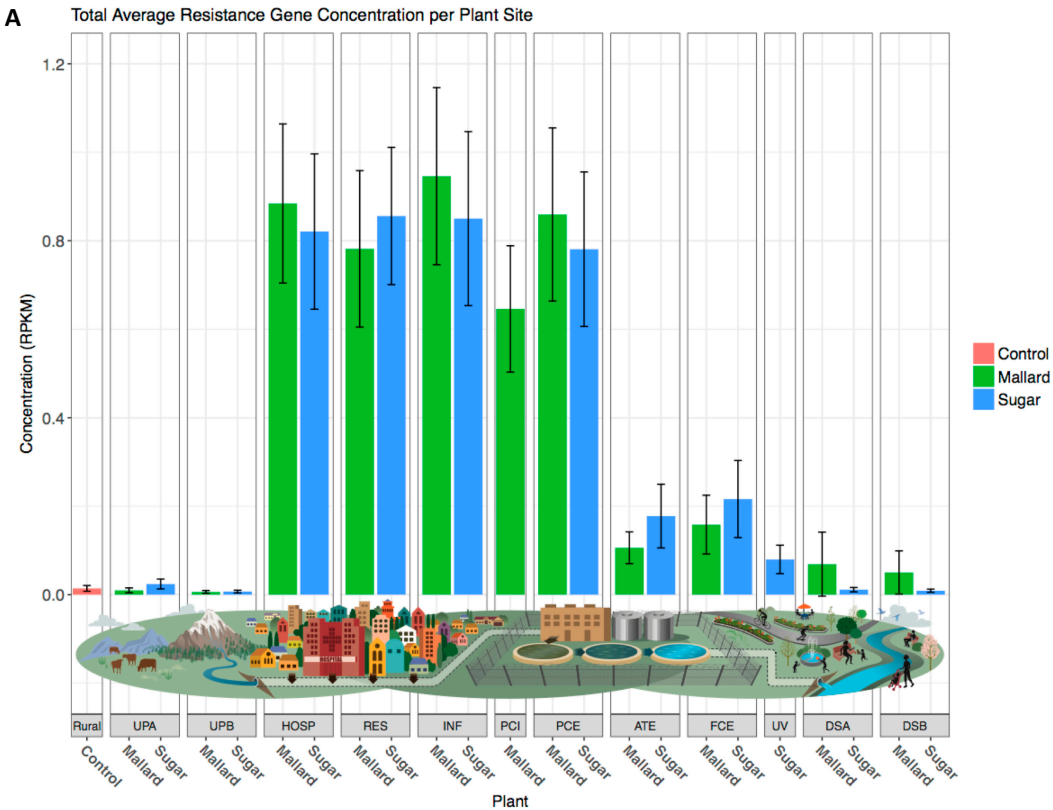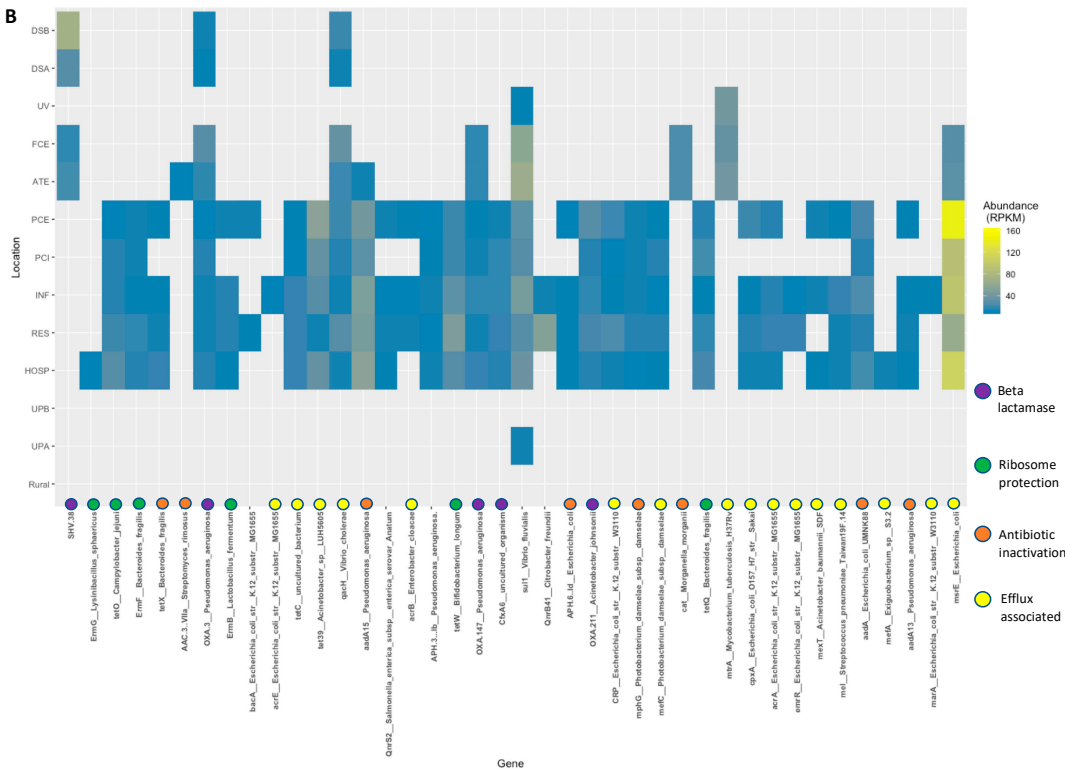
Figure 7. Abundance and differential abundance of antibiotic resistance genes. (A) The average number of antibiotic resistance-associated genetic elements across all four time points are reported in RPKM for each different site and each treatment plant. Standard deviations are shown within each bar. Rural sites are displayed in red, with Mallard and Little Sugar Creek sites in green and blue respectively. PCI is exclusive to Mallard, while UV is only in the Sugar Creek facility as denoted in Methods. (B) Relative abundance of antibiotic resistance elements. The top 40 average antibiotic resistance genes across all sampling timepoints with an average normalized RPKM of 10 or above are shown for each sampling site. Colored dots indicate common resistance mechanisms conferred by groups of similar resistance genes.

4. Discussion

In this survey of two Charlotte NC urban waterways and their associated treatment plants, we made four key observations. Each of these suggests targets for future research or innovation in water treatment methodology.

First, we assayed the concentration of 10 antibiotic compounds representing families of commonly used antibiotics, and found that multiple antibiotic concentrations are elevated downstream of wastewater treatment plants. Antibiotic compounds tend to be concentrated inside the plants relative to upstream and influent concentrations, whole compounds persist throughout the treatment cycle, and elevated concentrations of antibiotics are observed in moving waters downstream of the treatment plants. Both the interior sites in the plant and the downstream sites are environments that facilitate simultaneous exposure of bacteria to multiple antibiotics. Trunk lines handling hospital waste did not have significantly higher antibiotic levels than purely residential trunk lines.

Second, we assayed microbial communities at the same sites within treatment plants and stream watersheds, and found that treated water microbial communities become more like fresh water communities as waste progresses through the treatment process. Some antibiotic resistance terms originating from priority pathogens were observed in low relative abundance in environmental sites and at much higher relative abundance within the influent and in the treatment plants. Surprisingly, influent from hospital-associated and all-residential neighborhoods was not significantly different

from the microbial community perspective. We observed increased relative abundance of expected microbial signatures, such as human gut associated Bacteroidetes and Firmicutes and activated sludge associated taxa, in downstream waters. Levels of these attenuated between the proximal and distal downstream sites and did not significantly change the core microbial community of the streamwater. More downstream sampling locations would be required to accurately model this trend, and it will be of interest to assay antibiotic levels at the endpoint of the Catawba and Yadkin Pee-Dee rivers in southeast coastal waters as well.

Third, we analyzed relative abundance of functional pathways in the stream and treatment plant microbial communities, and found that shifts in stream microbial community function are observed downstream of treated water release points. Here, we focused primarily on the impact of water release on the function of the stream community. Comparing upstream and downstream sites, we observed that some core functions of stream microbes, including many pathway terms for DNA and peptide recycling and biosynthesis, were suppressed relative to upstream sites. This is an interesting finding that we cannot yet explain mechanistically, but it suggests that further investigation of sediments in urban streams under long-term antibiotic stress is necessary to understand the mechanism of impact of this stressor on community function.

Finally, we analyzed presence of antibiotic resistance gene (ARG) signatures in the plant and stream microbial communities, and found that resistance genes are slightly more abundant downstream of wastewater treatment plants. This was not a foregone conclusion of this study, because in other contexts ARG signatures have been observed to increase downstream of treated water release. However, Charlotte Water's treatment process, which includes UV treatment of waters prior to final release, appears to be very effective in reducing ARG relative abundance. While certain ARG signatures, including concerning carbapenem resistance and broad spectrum beta lactamases, originating from organisms such as *Pseudomonas aeruginosa*, *Vibrio cholerae*, and *Escherichia coli* were detectable in low relative abundance downstream of the plants, the reduction in relative abundance of these signatures relative to influent and internal WWTP locations was dramatic, especially considering that the testing that Charlotte Water conducts for pathogens in treated water is limited to the standard fecal coliform test.

Testing for antibiotics and associated resistance genes is not conducted as part of the wastewater treatment and monitoring process at Charlotte Water, nor is this implemented as standard practice in any known treatment facilities in the United States. However, the spread of resistance to common antibiotics is a well-known public health threat, and the presence of antibiotics in sub-lethal concentrations is known to drive microbial evolution [15,16,58]. Based on available knowledge about dissemination of pharmaceutical compounds in surface waters in North America [59], we hypothesized that either the processing or the release of treated water could create a condition where mixtures of bacteria are simultaneously exposed to multiple antibiotics, and facilitate the spread of antibiotic resistance [60]. We observed that conditions for multiple antibiotic exposure exist inside the water treatment plants as well as at downstream urban locations.

Wastewater treatment plant influent is known to contain significant populations of human gut associated pathogens, and regional sewage microbial profiles are tightly correlated with the microbial profiles of human residents in the region [61]. Although we observe in this study that gut-associated taxa are somewhat elevated in downstream waters, the microbial profile of moving waters in upstream sites in the city is not far removed from the profiles of more remote rural sites, largely devoid of most resistance elements. The addition of treated water to the stream appears as a temporary perturbation in the microbial community that begins to attenuate further away from the release point. This likely allows the stream microbial profile to return to its pre-effluent baseline within a relatively short distance, particularly with the limited downstream population effects reported here. Recent works describe similar changes in stream biofilms [34], although the extent of observable species changes was less significant in our samples.

Charlotte's WWTPs accumulate significant quantities and varieties of genetic elements associated with antibiotic resistance, but unlike some other treatment facilities [62], are quite effective in removing them prior to release. Simultaneous exposures of microbes to multiple antibiotics within

556 the plants themselves, where antibiotic concentrations are highest, are relatively short, although a
557 typical residence time of approximately 20 days still encompasses many microbial generations.
558 However, residence times for microbes are much longer in downstream sites, especially in stream
559 sediments and biofilms. We showed that the release of treated water maintains significantly elevated
560 concentrations of multiple antibiotics in the stream for a significant distance downstream of release.
561 The very effective removal of ARG gene signatures by Charlotte Water's treatment process suggests
562 that even though the influent, and the interior of the plant itself, is a diverse hothouse of pathogens
563 and antibiotic resistance, those elements escaping the plant is not likely to be the main mechanism of
564 treated water impact on downstream microbial ecology [63]. Only a small number of recognizable
565 ARGs pass through the water treatment process to end up at detectable levels in the streams. This is
566 not uniformly true in modern water treatment systems, as demonstrated in another recent study [33].
567 Rather, we now hypothesize that the persistently elevated concentration of antibiotics downstream
568 of treated water release may be creating its own microenvironment, and that this will impact the
569 function of stream sediment communities and their capacity to provide ecosystem services. Surface
570 waters in areas devoid of anthropogenic involvement are known to be devoid of most antibiotic
571 resistance markers, including many mobile elements [64,65]; however, some resistance factors do
572 emerge naturally through the process of recombination and mutation [64], and the conditions with
573 the potential to exacerbate this process do exist in Charlotte's urban streams [66].   Further study of
574 the impact of maintaining permanent low concentrations of antibiotics in the stream, in the presence
575 of resistance elements from wastewater or environmental sources, is critical for understanding how
576 we can improve water treatment to safeguard both ecosystem and human health.

610   **Conflicts of Interest:** The authors declare no conflict of interest.

611   **References**

612   1.   Walsh, C.J.; Roy, A.H.; Feminella, J.W.; Cottingham, P.D.; Groffman, P.M.; II, R.P.M. The
613   urban stream syndrome: Current knowledge and the search for a cure. *J. N. Am. Benthol. Soc.*
614   **2005**, *24*, 706-723.

615   2.   Yu, S.; Wu, Q.; Li, Q.; Gao, J.; Lin, Q.; Ma, J.; Xu, Q.; Wu, S. Anthropogenic land uses elevate
616   metal levels in stream water in an urbanizing watershed. *Sci. Total Environ.* **2014**, *488-489*, 61-
617   69.

618   3.   Fisher, J.C.; Newton, R.J.; Dila, D.K.; McLellan, S.L. Urban microbial ecology of a freshwater
619   estuary of lake michigan. *Elementa (Wash D C)* **2015**, *3*.

620   4.   Qu, X.; Ren, Z.; Zhang, H.; Zhang, M.; Zhang, Y.; Liu, X.; Peng, W. Influences of
621   anthropogenic land use on microbial community structure and functional potentials of
622   stream benthic biofilms. *Scientific Reports* **2017**, *7*, 15117.

623   5.   Newton, R.J.; McLellan, S.L. A unique assemblage of cosmopolitan freshwater bacteria and
624   higher community diversity differentiate an urbanized estuary from oligotrophic lake
625   michigan. *Front Microbiol* **2015**, *6*, 1028.

626   6.   McLellan, S.L.; Fisher, J.C.; Newton, R.J. The microbiome of urban waters. *Int. Microbiol.* **2015**,
627   *18*, 141-149.

628   7.   Kasprzyk-Hordern, B.; Dinsdale, R.M.; Guwy, A.J. The removal of pharmaceuticals, personal
629   care products, endocrine disruptors and illicit drugs during wastewater treatment and its
630   impact on the quality of receiving waters. *Water Res.* **2009**, *43*, 363-380.

631   8.   Niemuth, N.J.; Jordan, R.; Crago, J.; Blanksma, C.; Johnson, R.; Klaper, R.D. Metformin
632   exposure at environmentally relevant concentrations causes potential endocrine disruption
633   in adult male fish. *Environ. Toxicol. Chem.* **2015**, *34*, 291-296.

634   9.   Conley, J.M.; Evans, N.; Mash, H.; Rosenblum, L.; Schenck, K.; Glassmeyer, S.; Furlong, E.T.;
635   Kolpin, D.W.; Wilson, V.S. Comparison of in vitro estrogenic activity and estrogen
636   concentrations in source and treated waters from 25 u.S. Drinking water treatment plants.
637   *Sci. Total Environ.* **2017**, *579*, 1610-1617.

638   10.   Beresford, N.; Baynes, A.; Kanda, R.; Mills, M.R.; Arias-Salazar, K.; Collins, T.J.; Jobling, S.
639   Use of a battery of chemical and ecotoxicological methods for the assessment of the efficacy
640   of wastewater treatment processes to remove estrogenic potency. *Journal of Visualized*
641   *Experiments : JoVE* **2016**, 54243.

642   11.   Zhang, Y.; Zhang, T.; Guo, C.; Lv, J.; Hua, Z.; Hou, S.; Zhang, Y.; Meng, W.; Xu, J. Drugs of
643   abuse and their metabolites in the urban rivers of beijing, china: Occurrence, distribution,
644   and potential environmental risk. *Sci. Total Environ.* **2017**, *579*, 305-313.

645   12.   Li, N.; Sheng, G.P.; Lu, Y.Z.; Zeng, R.J.; Yu, H.Q. Removal of antibiotic resistance genes from
646   wastewater treatment plant effluent by coagulation. *Water Res.* **2017**, *111*, 204-212.

647   13.   Prasse, C.; Schlüsener, M.P.; Schulz, R.; Ternes, T.A. Antiviral drugs in wastewater and
648   surface waters: A new pharmaceutical class of environmental relevance? *Environ. Sci. Technol.*
649   **2010**, *44*, 1728-1735.

650   14.   He, K.; Soares, A.D.; Adejumo, H.; McDiarmid, M.; Squibb, K.; Blaney, L. Detection of a wide
651   variety of human and veterinary fluoroquinolone antibiotics in municipal wastewater and
652   wastewater-impacted surface water. *J. Pharm. Biomed. Anal.* **2015**, *106*, 136-143.

653   15.   Andersson, D.I.; Hughes, D. Evolution of antibiotic resistance at non-lethal drug
654   concentrations. *Drug Resistance Updates* **2012**, *15*, 162-172.
655   16.   Hughes, D.; Andersson, D.I. Selection of resistance at lethal and non-lethal antibiotic
656   concentrations. *Curr. Opin. Microbiol.* **2012**, *15*, 555-560.
657   17.   Ma, L.; Xia, Y.; Li, B.; Yang, Y.; Li, L.-G.; Tiedje, J.M.; Zhang, T. Metagenomic assembly reveals
658   hosts of antibiotic resistance genes and the shared resistome in pig, chicken, and human feces.
659   *Environ. Sci. Technol.* **2016**, *50*, 420-427.
660   18.   Call, D.R.; Matthews, L.; Subbiah, M.; Liu, J. Do antibiotic residues in soils play a role in
661   amplification and transmission of antibiotic resistant bacteria in cattle populations? *Frontiers*
662   *in Microbiology* **2013**, *4*, 193.
663   19.   Wang, F.-H.; Qiao, M.; Su, J.-Q.; Chen, Z.; Zhou, X.; Zhu, Y.-G. High throughput profiling of
664   antibiotic resistance genes in urban park soils with reclaimed water irrigation. *Environ. Sci.*
665   *Technol.* **2014**, *48*, 9079-9085.
666   20.   Fahrenfeld, N.; Ma, Y.; O'Brien, M.; Pruden, A. Reclaimed water as a reservoir of antibiotic
667   resistance genes: Distribution system and irrigation implications. *Frontiers in Microbiology*
668   **2013**, *4*, 130.
669   21.   Pruden, A. Balancing water sustainability and public health goals in the face of growing
670   concerns about antibiotic resistance. *Environ. Sci. Technol.* **2014**, *48*, 5-14.
671   22.   Pehrsson, E.C.; Tsukayama, P.; Patel, S.; Mejia-Bautista, M.; Sosa-Soto, G.; Navarrete, K.M.;
672   Calderon, M.; Cabrera, L.; Hoyos-Arango, W.; Bertoli, M.T., *et al.* Interconnected
673   microbiomes and resistomes in low-income human habitats. *Nature* **2016**, *533*, 212-216.
674   23.   LaPara, T.M.; Burch, T.R.; McNamara, P.J.; Tan, D.T.; Yan, M.; Eichmiller, J.J. Tertiary-treated
675   municipal wastewater is a significant point source of antibiotic resistance genes into duluth-
676   superior harbor. *Environ. Sci. Technol.* **2011**, *45*, 9543-9549.
677   24.   Munck, C.; Albertsen, M.; Telke, A.; Ellabaan, M.; Nielsen, P.H.; Sommer, M.O. Limited
678   dissemination of the wastewater treatment plant core resistome. *Nat Commun* **2015**, *6*, 8452.
679   25.   Christgen, B.; Yang, Y.; Ahammad, S.Z.; Li, B.; Rodriquez, D.C.; Zhang, T.; Graham, D.W.
680   Metagenomics shows that low-energy anaerobic−aerobic treatment reactors reduce antibiotic
681   resistance gene levels from domestic wastewater. *Environ. Sci. Technol.* **2015**, *49*, 2577-2584.
682   26.   Segata, N.; Waldron, L.; Ballarini, A.; Narasimhan, V.; Jousson, O.; Huttenhower, C.
683   Metagenomic microbial community profiling using unique clade-specific marker genes. *Nat.*
684   *Methods* **2012**, *9*, 811-814.
685   27.   Team, R.C. *R: A language and environment for statistical computing*, 2017.
686   28.   Wickham, H. Ggplot2: Elegant graphics for data analysis. *Springer-Verlag* **2009**.
687   29.   Kaminski, J.; Gibson, M.K.; Franzosa, E.A.; Segata, N.; Dantas, G.; Huttenhower, C. High-
688   specificity targeted functional profiling in microbial communities with shortbred. *PLoS*
689   *Comput Biol* **2015**, *11*, e1004557.
690   30.   McArthur, A.G.; Waglechner, N.; Nizam, F.; Yan, A.; Azad, M.A.; Baylay, A.J.; Bhullar, K.;
691   Canova, M.J.; De Pascale, G.; Ejim, L., *et al.* The comprehensive antibiotic resistance database.
692   *Antimicrob. Agents Chemother.* **2013**, *57*, 3348-3357.
693   31.   Suzek, B.E.; Wang, Y.; Huang, H.; McGarvey, P.B.; Wu, C.H.; the UniProt, C. Uniref clusters:
694   A comprehensive and scalable alternative for improving sequence similarity searches.
695   *Bioinformatics* **2015**, *31*, 926-932.

696   32.   Benjamini, Y.; Hochberg, Y. Controlling the false discovery rate - a practical and powerful
697   approach to multiple testing. *Journal of the Royal Statistical Society Series B-Methodological* **1995**,
698   *57*, 289-300.
699   33.   Wang, M.; Shen, W.; Yan, L.; Wang, X.H.; Xu, H. Stepwise impact of urban wastewater
700   treatment on the bacterial community structure, antibiotic contents, and prevalence of
701   antimicrobial resistance. *Environ. Pollut.* **2017**.
702   34.   Aubertheau, E.; Stalder, T.; Mondamert, L.; Ploy, M.C.; Dagot, C.; Labanowski, J. Impact of
703   wastewater treatment plant discharge on the contamination of river biofilms by
704   pharmaceuticals and antibiotic resistance. *Sci. Total Environ.* **2017**, *579*, 1387-1398.
705   35.   Zhang, J.; Yang, Y.; Zhao, L.; Li, Y.; Xie, S.; Liu, Y. Distribution of sediment bacterial and
706   archaeal communities in plateau freshwater lakes. *Appl. Microbiol. Biotechnol.* **2015**, *99*, 3291-
707   3302.
708   36.   Yang, C.; Zhang, W.; Liu, R.; Li, Q.; Li, B.; Wang, S.; Song, C.; Qiao, C.; Mulchandani, A.
709   Phylogenetic diversity and metabolic potential of activated sludge microbial communities in
710   full-scale wastewater treatment plants. *Environ. Sci. Technol.* **2011**, *45*, 7408-7415.
711   37.   McLellan, S.L.; Huse, S.M.; Mueller-Spitz, S.R.; Andreishcheva, E.N.; Sogin, M.L. Diversity
712   and population structure of sewage-derived microorganisms in wastewater treatment plant
713   influent. *Environ. Microbiol.* **2010**, *12*, 378-392.
714   38.   MacRae, J.D.; Smit, J. Characterization of caulobacters isolated from wastewater treatment
715   systems. *Appl. Environ. Microbiol.* **1991**, *57*, 751-758.
716   39.   Lücker, S.; Wagner, M.; Maixner, F.; Pelletier, E.; Koch, H.; Vacherie, B.; Rattei, T.; Damsté,
717   J.S.S.; Spieck, E.; Le Paslier, D., *et al.* A metagenome illuminates the physiology and evolution
718   of globally important nitrite-oxidizing bacteria. *Proceedings of the National Academy of Sciences*
719   **2010**, *107*, 13479.
720   40.   Kim, M.K.; Choi, K.M.; Yin, C.R.; Lee, K.Y.; Im, W.T.; Lim, J.H.; Lee, S.T. Odorous swine
721   wastewater treatment by purple non-sulfur bacteria, rhodopseudomonas palustris, isolated
722   from eutrophicated ponds. *Biotechnol. Lett.* **2004**, *26*, 819-822.
723   41.   Gonzalez-Martinez, A.; Sihvonen, M.; Muñoz-Palazon, B.; Rodriguez-Sanchez, A.; Mikola,
724   A.; Vahala, R. Microbial ecology of full-scale wastewater treatment systems in the polar arctic
725   circle: Archaea, bacteria and fungi. *Scientific Reports* **2018**, *8*, 2208.
726   42.   Saunders, A.M.; Albertsen, M.; Vollertsen, J.; Nielsen, P.H. The activated sludge ecosystem
727   contains a core community of abundant organisms. *ISME J* **2016**, *10*, 11-20.
728   43.   Balcom, I.N.; Driscoll, H.; Vincent, J.; Leduc, M. Metagenomic analysis of an ecological
729   wastewater treatment plant's microbial communities and their potential to metabolize
730   pharmaceuticals. *F1000Res* **2016**, *5*, 1881.
731   44.   Medhi, K.; Thakur, I.S. Bioremoval of nutrients from wastewater by a denitrifier paracoccus
732   denitrificans istod1. *Bioresource Technology Reports* **2018**, *1*, 56-60.
733   45.   Pagnier, I.; Croce, O.; Robert, C.; Raoult, D.; La Scola, B. Genome sequence of reyranella
734   massiliensis, a bacterium associated with amoebae. *J. Bacteriol.* **2012**, *194*, 5698.
735   46.   Pehl, M.J.; Jamieson, W.D.; Kong, K.; Forbester, J.L.; Fredendall, R.J.; Gregory, G.A.;
736   McFarland, J.E.; Healy, J.M.; Orwin, P.M. Genes that influence swarming motility and biofilm
737   formation in variovorax paradoxus eps. *PloS one* **2012**, *7*, e31832.

738 47. Kampfer, P.; Witzenberger, R.; Denner, E.B.; Busse, H.J.; Neef, A. Sphingopyxis witflariensis
739   sp. Nov., isolated from activated sludge. *Int. J. Syst. Evol. Microbiol.* **2002**, *52*, 2029-2034.

740 48. Zielinska, M.; Rusanowska, P.; Jarzabek, J.; Nielsen, J.L. Community dynamics of
741   denitrifying bacteria in full-scale wastewater treatment plants. *Environ. Technol.* **2016**, *37*,
742   2358-2367.

743 49. Cai, L.; Ju, F.; Zhang, T. Tracking human sewage microbiome in a municipal wastewater
744   treatment plant. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 3317-3326.

745 50. Jiang, K.; Sanseverino, J.; Chauhan, A.; Lucas, S.; Copeland, A.; Lapidus, A.; Del Rio, T.G.;
746   Dalin, E.; Tice, H.; Bruce, D., *et al.* Complete genome sequence of thauera aminoaromatica
747   strain mz1t. *Stand Genomic Sci* **2012**, *6*, 325-335.

748 51. Al Atrouni, A.; Joly-Guillou, M.L.; Hamze, M.; Kempf, M. Reservoirs of non-baumannii
749   acinetobacter species. *Front Microbiol* **2016**, *7*, 49.

750 52. Chagas, T.P.; Seki, L.M.; Cury, J.C.; Oliveira, J.A.; Davila, A.M.; Silva, D.M.; Asensi, M.D.
751   Multiresistance, beta-lactamase-encoding genes and bacterial diversity in hospital
752   wastewater in rio de janeiro, brazil. *J. Appl. Microbiol.* **2011**, *111*, 572-581.

753 53. Carr, E.L.; Eales, K.L.; Seviour, R.J. Substrate uptake by gordonia amarae in activated sludge
754   foams by fish-mar. *Water Sci. Technol.* **2006**, *54*, 39-45.

755 54. Hanada, S.; Liu, W.T.; Shintani, T.; Kamagata, Y.; Nakamura, K. Tetrasphaera elongata sp.
756   Nov., a polyphosphate-accumulating bacterium isolated from activated sludge. *Int. J. Syst.*
757   *Evol. Microbiol.* **2002**, *52*, 883-887.

758 55. Zhang, B.; Xu, X.; Zhu, L. Structure and function of the microbial consortia of activated sludge
759   in typical municipal wastewater treatment plants in winter. *Scientific Reports* **2017**, *7*, 17930.

760 56. Abubucker, S.; Segata, N.; Goll, J.; Schubert, A.M.; Izard, J.; Cantarel, B.L.; Rodriguez-
761   Mueller, B.; Zucker, J.; Thiagarajan, M.; Henrissat, B., *et al.* Metabolic reconstruction for
762   metagenomic data and its application to the human microbiome. *PLoS Comput Biol* **2012**, *8*,
763   e1002358.

764 57. Caspi, R.; Altman, T.; Billington, R.; Dreher, K.; Foerster, H.; Fulcher, C.A.; Holland, T.A.;
765   Keseler, I.M.; Kothari, A.; Kubo, A., *et al.* The metacyc database of metabolic pathways and
766   enzymes and the biocyc collection of pathway/genome databases. *Nucleic Acids Res.* **2014**, *42*,
767   D459-471.

768 58. Andersson, D.I.; Hughes, D. Microbiological effects of sublethal levels of antibiotics. *Nat. Rev.*
769   *Microbiol.* **2014**, *12*, 465-478.

770 59. Kolpin, D.W.; Furlong, E.T.; Meyer, M.T.; Thurman, E.M.; Zaugg, S.D.; Barber, L.B.; Buxton,
771   H.T. Pharmaceuticals, hormones, and other organic wastewater contaminants in u.S.
772   Streams, 1999–2000: A national reconnaissance. *Environ. Sci. Technol.* **2002**, *36*, 1202-1211.

773 60. Rodriguez-Rojas, A.; Rodriguez-Beltran, J.; Couce, A.; Blazquez, J. Antibiotics and antibiotic
774   resistance: A bitter fight against evolution. *Int. J. Med. Microbiol.* **2013**, *303*, 293-297.

775 61. Newton, R.J.; McLellan, S.L.; Dila, D.K.; Vineis, J.H.; Morrison, H.G.; Eren, A.M.; Sogin, M.L.
776   Sewage reflects the microbiomes of human populations. *MBio* **2015**, *6*, e02574.

777 62. Yoon, Y.; Chung, H.J.; Wen Di, D.Y.; Dodd, M.C.; Hur, H.G.; Lee, Y. Inactivation efficiency
778   of plasmid-encoded antibiotic resistance genes during water treatment with chlorine, uv, and
779   uv/h2o2. *Water Res.* **2017**, *123*, 783-793.

780   63.   Laquaz, M.; Dagot, C.; Bazin, C.; Bastide, T.; Gaschet, M.; Ploy, M.C.; Perrodin, Y. Ecotoxicity
781   and antibiotic resistance of a mixture of hospital and urban sewage in a wastewater treatment
782   plant. *Environ Sci Pollut Res Int* **2017**.
783   64.   Chen, B.; Yuan, K.; Chen, X.; Yang, Y.; Zhang, T.; Wang, Y.; Luan, T.; Zou, S.; Li, X.
784   Metagenomic analysis revealing antibiotic resistance genes (args) and their genetic
785   compartments in the tibetan environment. *Environ. Sci. Technol.* **2016**, *50*, 6670-6679.
786   65.   Pawlowski, A.C.; Wang, W.; Koteva, K.; Barton, H.A.; McArthur, A.G.; Wright, G.D. A
787   diverse intrinsic antibiotic resistome from a cave bacterium. *Nat Commun* **2016**, *7*, 13803.
788   66.   Li, B.; Ju, F.; Cai, L.; Zhang, T. Profile and fate of bacterial pathogens in sewage treatment
789   plants revealed by high-throughput metagenomic approach. *Environ. Sci. Technol.* **2015**, *49*,
790   10492-10502.