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# Survivor Microbial Populations in Post-Chlorinated Wastewater are Strongly Associated with Untreated Hospital Sewage and Include Ceftazidime and Meropenem Resistant Populations

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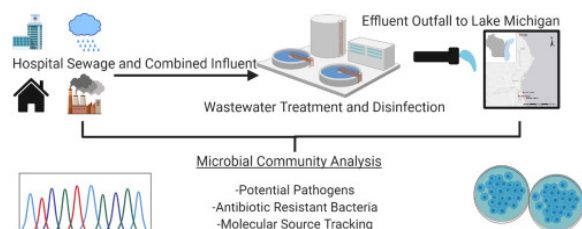
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## Abstract

Wastewater treatment plant (WWTP) effluent has been implicated in the spread of antibiotic resistant bacteria (ARB), including pathogens, as the WWTP environment contains multiple selective pressures that may increase mutation rates, pathogen survivability, and induce gene transfer between bacteria. In WWTPs receiving hospital sewage, this selective effect may be more pronounced due to increased concentrations of antibiotics, ARB, and clinical pathogens from hospital sewage. To determine the extent to which hospital sewage contributes to the microbial community of disinfected wastewater which is released into the environment, we used 16S rRNA sequencing of hospital sewage, WWTP influent, primary effluent, Post-Chlorinated Effluent, and receiving sediments in a combined sewage system to track changes in microbial community composition. We also sequenced the culturable survivor community resistant to  $\beta$ -lactam antibiotics within disinfected effluent. Using molecular source tracking, we found that the hospital sewage microbiome contributes an average of 11.49% of the microbial community in Post-Chlorinated Effluents, suggesting microorganisms identified within hospital sewage can survive or are enriched by the chlorination disinfection process. Additionally, we identified 28 potential pathogens to the species level, seven of which remained detectable in Post-Chlorinated Effluent and environmental sediments. When Post-Chlorinated Effluents were cultured on media containing  $\beta$ -lactam antibiotics ceftazidime and meropenem, a diverse antibiotic resistant survivor community was identified including potential human pathogens *Bacillus cereus*, *Bacillus pumilus*, and *Chryseobacterium indologenes*. Together, these results indicate that although wastewater treatment does significantly reduce pathogenic loads and ARBs, their continual presence in disinfected wastewater and receiving sediments suggests additional treatment and microbial tracking systems are needed to reduce human and animal health risks.

### Graphical abstract



## Keywords

Wastewater treatment, Hospital sewage,  $\beta$ -Lactam resistance, Next-generation sequencing, Microbial source tracking

## 1. Introduction

Wastewater treatment is the primary source of pollution reduction for industrial, hospital, and sanitary waste that would otherwise impair surface waters (USEPA, 2004a, USEPA, 2004b). A variety of chemical and biological pollutants enter wastewater treatment plants (WWTPs) including, but not limited to, pharmaceuticals, heavy-metals, detergents, pathogens, and antibiotic resistant bacteria (ARB) (Anjali and Shanthakumar, 2019; USEPA, 2004a, USEPA, 2004b). Insufficient removal of wastewater pollutants impacts both environmental (Naidoo and Olaniran, 2013; Wakelin et al., 2008) and human health (Kim and Aga, 2007), especially in regions where WWTP discharge intermixes with drinking water sources such as the Great Lakes region of the United States (Blair et al., 2013; Sedmak et al., 2005). Monitoring chemical and biological contaminants is vital in these regions, but monitoring programs generally focus on a limited number of specific pollutants such as fecal indicators or nitrates (USEPA, 2004a, USEPA, 2004b). Due to the global rise in antibiotic resistance in both the clinic and environment, additional emphasis is now being placed on the detection and removal of pharmaceuticals, ARB and antibiotic resistance genes (ARGs), and pathogens from treated wastewater (Al-Gheethi et al., 2018; Jäger et

al., 2018; Luddeke et al., 2015; Ventola, 2015) but federal and state monitoring programs for these pollutants remains minimal or nonexistent (Weinberg et al., 2005).

In recent years, WWTP effluent has been repeatedly implicated in the spread of antibiotic resistance and pathogenic bacteria (Al-Jassim et al., 2015; Sharma et al., 2016), as the WWTP environment contains multiple selective pressures that may increase mutation rates or induce gene transfer between bacteria (Ju et al., 2019; Rizzo et al., 2013). Additionally, elevated concentrations of antibiotics and ARGs in WWTP effluent and receiving waters have been found in multiple studies (Aubertheau et al., 2017; Hultman et al., 2018; Karkman et al., 2016). In WWTPs receiving hospital sewage, this effect may be more pronounced due to increased concentrations of antibiotics, ARB, and clinical pathogens entering the treatment system from hospital sewage (Amador et al., 2015; Khan et al., 2019; Korzeniewska and Harnisz, 2018). Reduction rates of antibiotics and ARG in WWTPs vary, but full reduction in treated effluent is rare (Hiller et al., 2019; Laht et al., 2014). As the number of resistant bacterial infections continues to rise worldwide, wastewater treatment processes that focus on the removal of antibiotics of critical importance for human health, including cephalosporins and carbapenems, is necessary to reduce the selection and spread of bacteria resistant to these antibiotics (WHO, 2019).

Although animal and human pathogen removal is a primary goal of wastewater treatment (USEPA, 2004a, USEPA, 2004b), monitoring the diverse array of potential pathogens entering WWTPs is not feasible. Thus, indicator organisms including *Escherichia coli* and fecal coliforms are routinely used to identify pathogen risk in treated effluent; however, correlations between these indicator organisms and other pathogenic species in WWTP effluent are weak (Edge et al., 2013; Osuolale and Okoh, 2017). Because the presence of pathogens (other than fecal indicators) within the WWTP is not routinely tested, determining the source of these microorganisms throughout the treatment process and in downstream environmental samples is difficult but is necessary to reduce environmental and public health risks.

Characterizing the microbial ecology of the wastewater treatment system from untreated influents to receiving freshwater ecosystems allows for the characterization of microbial populations that survive wastewater treatment and disinfection processes, including ARB and potential pathogens. Recent studies of WWTP microbial community structure have primarily used culture-independent molecular techniques, specifically Next Generation 16S rRNA sequencing to identify taxonomic diversity (Fisher et al., 2015; Price et al., 2018) or shotgun metagenomics to identify functional genes (Tang et al., 2016). However, these molecular approaches alone cannot provide information on the viable subset of the community that is resistant to antibiotics following wastewater treatment and disinfection. Here, we have combined a deep sequencing approach with traditional culture-based assays to identify both: 1) The total microbial community (including potential pathogens) of the influent sources and treated effluents of a WWTP receiving combined hospital and municipal waste in Milwaukee, Wisconsin; and 2) The viable subset of the microbial community in treated and disinfected wastewater that is resistant to critically important antibiotics. By combining these two approaches, we can assess the contribution of hospital sewage and municipal sewage to the final microbial community composition of disinfected wastewater and receiving freshwater sediments, an area which remains largely understudied. In addition, by culturing survivor bacteria from disinfected wastewater on media containing antibiotics and following with Next Generation 16S rRNA sequencing, we identify the diversity of antibiotic resistant survivor bacteria in treated effluent. This study allows us to consider the environmental and human health implications of the diverse microorganisms that survive wastewater treatment and persist in the environment, in addition to providing possible avenues for mitigation of harmful bacteria during the WWTP process.

## 2. Materials and methods

### 2.1. Sample collection and DNA extraction

Replicate grab samples (2 samples per collection timepoint) from WWTP influent, pre-chlorinated (primary) effluent, and Post-Chlorinated Effluent were collected from the Jones Island Water Reclamation Facility (JI) serving the metropolitan area of Milwaukee, WI. The plant services the city of Milwaukee and neighboring communities constituting ~40 km<sup>2</sup> of combined sewer system, receiving a combination of sanitary sewage, stormwater, and hospital sewage with an average daily flow of 123 MGD and a maximum daily flow of 300 MGD (MMSD, 2020; USEPA, 2007). JI is an activated sludge wastewater treatment plant which includes preliminary treatment to remove large solids and trash, secondary activated sludge treatment to remove nutrients, including phosphorus, and biological contaminants, and disinfection with chlorine before being discharged into the Milwaukee Outer Harbor. Samples were collected weekly for three consecutive weeks during October 2018 to reduce the influence of seasonal variables on WWTP microbial community composition (Newton et al., 2015) to answer our primary research question: what is the source of bacterial populations in Post-Chlorinated Effluents? Samples (1 L, sterile amber glass bottles, Thermo Scientific™, Waltham, MA) were collected at approximately 10 a.m. on the 5th, 12th, and 18th October 2018 and were processed within one hour of collection.

Replicate grab hospital sewage samples (2 samples per timepoint per wastewater outflow, 1 L, sterile amber glass bottles, Thermo Scientific™, Waltham, MA) were also collected from two wastewater outflow locations at the Medical College of Wisconsin, Milwaukee, WI at approximately 7 a.m. on the 2nd, 11th, and 16th October 2018. Water entering the sewage system from within the hospital is pretreated by hospital unit with appropriate disinfectants (bleach, autoclave, UV, etc.); however, the collective hospital wastewater is not further pretreated as a whole before entering the combined sewage system serviced by JI. Both JI and hospital water samples (250 mL) were filtered through 0.2 µm Whatman Nuclepore polycarbonate filters (GE Healthcare, Chicago, IL) within one hour of collection and subsequently frozen at -20 °C until DNA extraction.

DNA was extracted from frozen filters using the PowerWater DNA Isolation Kit following manufacturer protocols (MoBio, Carlsbad, CA). Sediment grab samples collected in 2014–2015 from four locations in the Milwaukee Harbor were included in this study to determine the environmental microbial community composition downstream of the WWTP. Sediment sampling sites included Jones Island (immediately adjacent to the JI outflow site), Outer Harbor (approximately 0.5 km from the JI outflow site), Inner Harbor (highly polluted due to boat traffic and three major river inflows, but unimpacted by the JI outflow site), and Atwater (sediments upstream of municipal WWTP outflows in the greater Milwaukee area). See Fig. S1 for sediment sampling location details. Sediments serve as a natural sink and are excellent indicators of chronic pollution (Quero et al., 2015; Sutcliffe et al., 2019), thus we used these samples as a valid comparison of the impact of WWTP effluent on microbial community composition. DNA was extracted from 0.5 g of sediment per sample using the MoBio PowerSoil DNA Isolation Kit (Qiagen, Germantown, MD) following manufacturer's instructions.

### 2.2. DNA sequencing and amplicon sequence mapping

The Visualization and Analysis of Microbial Population Structure (VAMPS) universal primers (518F, 926R) (Huse et al., 2010) were used to amplify the V4-V5 hypervariable region of the 16S rRNA gene followed by Next Generation Sequencing (NGS). Paired-end sequencing of water DNA was performed by the Great Lakes Genomics Center (Milwaukee, WI) with the Illumina MiSeq Platform. Reads were processed using DADA2 v1.9.28 (Callahan et al., 2016) with default parameters. Briefly, primers were removed from the raw, demultiplexed reads, and only those reads with minimum base quality score of two were retained in subsequent steps. Forward and reverse reads were then truncated to 240 bp and 210 bp, respectively, and were only retained if maximum expected errors associated with such reads were equal to or less than two for forward and reverse

reads. Reads were error-corrected and pooled prior to inferring sample composition. Error-corrected reads were then merged to yield the complete sequence spanning the sequenced hypervariable region and chimeric sequences were removed. Taxonomy was assigned using the RDP classifier based on the SILVA database v132. Species-level identification was accomplished using exact string-matches against a customized variant of the same database (Callahan, 2018). The resulting amplicon sequence variant (ASV) table was further processed by removing any ASVs with less than four reads across all samples and/or ASVs present in less than two samples. Potential pathogens were identified at the species level using the DADA2 extension for 100% exact sequence matching of sample sequences to sequenced reference strains within the Silva database. To identify potential pathogens within each sample, the taxonomy assignment output file for all ASVs was first subset to 146 genera known to contain pathogens curated by previous studies (Fang et al., 2018; Li et al., 2015; Subirats et al., 2017). Next, those genera were subset again to only those ASVs mapped to species level using the strict 100% sequence matching criteria. Last, potential pathogens from each sample were identified from known human pathogenic species curated in the above studies followed by a confirmatory literature search of human infection cases with identified pathogenic species. Sequencing files are available in the National Center for Biotechnology Information Sequence Read Archive under BioProject ID Number PRJNA622864.

### 2.3. Colony forming unit (CFU) counts of *Escherichia coli* on mTEC chromogenic agar

Various volumes of water samples from the hospital sewage and each of the three compartments of JI were filtered on 0.2  $\mu$ M filters (Whatman® Nuclepore), plated in triplicate on mTEC chromogenic agar alone or mTEC agar containing the antibiotic ceftazidime (4  $\mu$ g/mL), incubated at 35 °C for 2 h, and subsequently incubated at 44.5 °C for 22 h. After incubation, magenta colonies were counted on plates containing between 30 and 300 colonies and reported as CFU/100 mL.

### 2.4. Recovery and DNA extraction of the culturable microbial community of Post-Chlorinated Effluent

Post-chlorinated wastewater from JI was collected and stored on ice until processed within one hour. Various volumes of wastewater were passed over 0.2  $\mu$ M filters and placed on either tryptic soy agar (TSA), TSA with meropenem (2  $\mu$ g/mL), or TSA with ceftazidime (2  $\mu$ g/mL) in triplicates. Plates were incubated at 35 °C and CFUs counted. Genomic DNA was isolated from triplicate filters of plates containing mostly isolated CFUs (60–250 CFUs/plate) using the FastDNA™ SPIN Kit for Soil (MP Biomedicals, Irvine, CA, USA). DNA was quantified using a nanodrop before processed for DNA sequencing as discussed above (Methods 4.2).

### 2.5. Statistical analyses

Statistical analyses were performed in R version 3.5.2. The phyloseq package in R was used to calculate relative abundances of raw ASV counts based on taxonomic classification used for downstream analyses (McMurdie and Holmes, 2013). To determine microbial community patterns based on  $\beta$ -diversity, a Bray-Curtis dissimilarity matrix of ASV relative abundance was produced in conjunction with permuted multivariate analysis of variance (PERMANOVA). Preferentially abundant taxa in each compartment of the wastewater treatment process were identified using linear discriminant analysis software LEfSe (v1.0) run on the Galaxy server with default parameters (Segata et al., 2011). Relative abundances of taxonomic count data agglomerated at the genus level were used as input. Molecular microbial source tracking was performed using fast expectation-maximization microbial source tracking (FEAST) (Shenhav et al., 2019) in R with default parameters to identify the potential contribution of Hospital Sewage and Combined Influent as source communities of the WWTP effluent microbial communities. This method is particularly useful at identifying microbial community composition similarities between source and sink communities and partitioning those values into potential contributions from each source. In this study, FEAST is based on the 16S rRNA relative abundance and taxonomic similarity between source and sink communities.

### 3. Results

#### 3.1. Microbial community composition is significantly different within separate compartments of the wastewater treatment process

Microbial community composition and diversity from wastewater samples collected from JI and the Medical College of Wisconsin was evaluated and included samples from hospital sewage (referred to as Hospital Sewage), combined hospital, municipal waste, and stormwater influent (referred to as Combined Influent), and treated effluent wastewater both before chlorination (referred to as Pre-Chlorinated Effluent) and after chlorination (referred to as Post-Chlorinated Effluent). The microbial community composition of freshwater receiving sediments was also analyzed as a representative sink for WWTP effluent pollution. Bray-Curtis dissimilarity of microbial community composition within the WWTP system were analyzed and found to be significantly different by sample type and sampling timepoint (PERMANOVA,  $p = .001$ , Table 1). Subsequent pairwise tests showed that samples from Hospital Sewage were significantly different across all sampling timepoints. Samples from Combined Influent were only significantly different on the first two sampling timepoints, and the Pre-Chlorinated Effluent and Post-Chlorinated Effluent were not significantly different regardless of sampling timepoint (Table 1). This result indicates that although wastewater entering the treatment system may be compositionally different, the treated wastewater exiting the WWTP is not significantly different over time. Our focus here is locational effects on microbial community composition, and as such the samples were pooled by sample type for downstream analyses.

Table 1. Comparison of microbial community composition beta diversity based on Bray-Curtis dissimilarity within the Jones Island Water reclamation plant by permuted MANOVA. Bolded values indicate significant differences ( $p < 0.05$ ).

<b>MANOVA</b>		
<b>Variable</b>	<b>Pseudo-F</b>	<b>P(MC)*</b>
Sample type	5.953	<b>0.001</b>
Sample timepoint	4.5457	<b>0.003</b>
Sample type × sample timepoint	4.6325	<b>0.001</b>
<b>MANOVA pairwise tests- Hospital Effluent by timepoint</b>		
<b>Groups</b>	<b>t</b>	<b>P(MC)</b>
Timepoint 1, 2	5.6403	<b>0.001</b>
Timepoint 1, 3	3.2529	<b>0.004</b>
Timepoint 2, 3	2.7687	<b>0.01</b>
<b>MANOVA pairwise tests- Combined Influent by timepoint</b>		
<b>Groups</b>	<b>t</b>	<b>P(MC)</b>
Timepoint 1, 2	2.6642	<b>0.032</b>
Timepoint 1, 3	3.1974	0.176
Timepoint 2, 3	2.743	0.226
<b>MANOVA pairwise tests- Pre-Chlorinated Effluent by timepoint</b>		
<b>Groups</b>	<b>t</b>	<b>P(MC)</b>
Timepoint 1, 2	2.0895	0.285
Timepoint 1, 3	1.0694	0.494
Timepoint 2, 3	1.6032	0.215
<b>MANOVA pairwise tests- Post-Chlorinated Effluent by timepoint</b>		
<b>Groups</b>	<b>t</b>	<b>P(MC)</b>
Timepoint 1, 2	1.7686	0.199
Timepoint 1, 3	1.3103	0.408
Timepoint 2, 3	7.8634	0.096

\*P(MC) = Permuted *P* value with Monte Carlo randomization.

Clear differences in the microbial community composition based on sample location within the wastewater treatment process are evident (Fig. 1) with significant differences in community composition between all compartments except Pre- and Post-Chlorinated Effluent samples (Table 1). The dominant microbial taxa of Hospital Sewage and Combined Influent samples include genera *Acinetobacter*, *Aeromonas*, *Arcobacter*, and *Bacteroides* while Pre- and Post-Chlorinated Effluents contained reduced numbers of these genera, particularly *Bacteroides* and *Acinetobacter* (Fig. 1). Treated Pre- and Post-Chlorinated Effluents were dominated by members of the genera *Acidovorax* and *Flavobacterium* along with higher *Pseudomonas* compared to Hospital Sewage and Combined Influent samples.

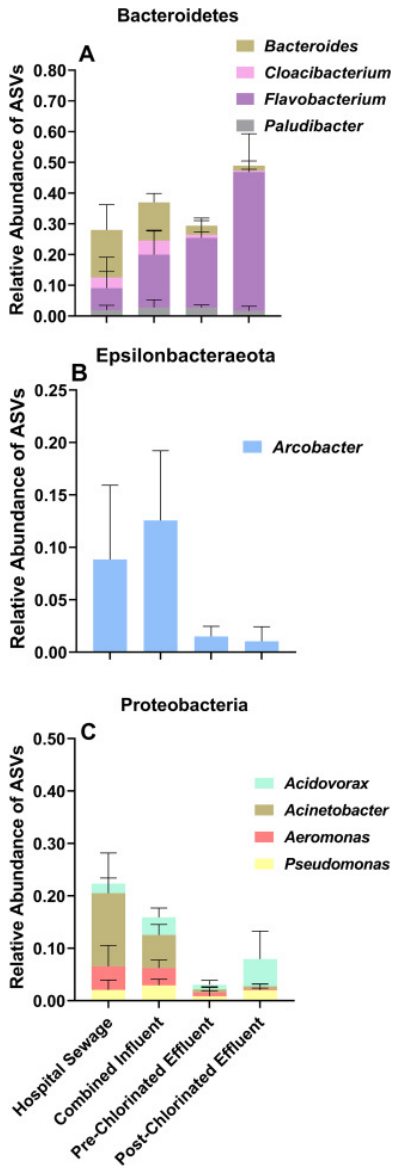


Fig. 1. Relative abundance of amplicon sequence variants (ASVs) in wastewater treatment compartments mapped to “highly abundant” genera, or those with a relative abundance of at least 1% across all samples presented as mean  $\pm$  SD. Replicate samples are combined by compartment.

Microorganisms are known to preferentially distribute within environments. Understanding the characteristic taxonomic composition of different compartments of the wastewater treatment process allows us to both



identify a microbial signature for each compartment and determine if transfer between compartments is occurring. We performed linear discriminant analysis using LEfSe to identify taxonomic biomarkers at the genus level within different compartments of the wastewater treatment system and found several biomarker genera including 32 within Hospital samples, 40 within Influent samples, 48 within Pre-Chlorinated Effluent samples, and 10 within Post-Chlorinated Effluent samples (Fig. S2). The dominant Hospital Sewage biomarker genera were *Acinetobacter*, *Bacteroides*, *Aeromonas* while the dominant Influent biomarker genera were *Arcobacter*, *Cloacibacterium*, and *Prevotella*. WWTP effluents were differentiated by biomarker taxa *Pedobacter*, *Aquabacterium*, and *Sediminibacterium* in Pre-Chlorinated Effluent, and *Flavobacterium*, *Rheinheimera*, and *Fluviicola* in Post-Chlorinated Effluent. The relative abundance of signature taxa from each compartment of the WWTP system is shown in Fig. 2. Although the relative abundance of biomarker taxa is highest in the specified WWTP compartment, they are frequently found in other compartments of the WWTP process.

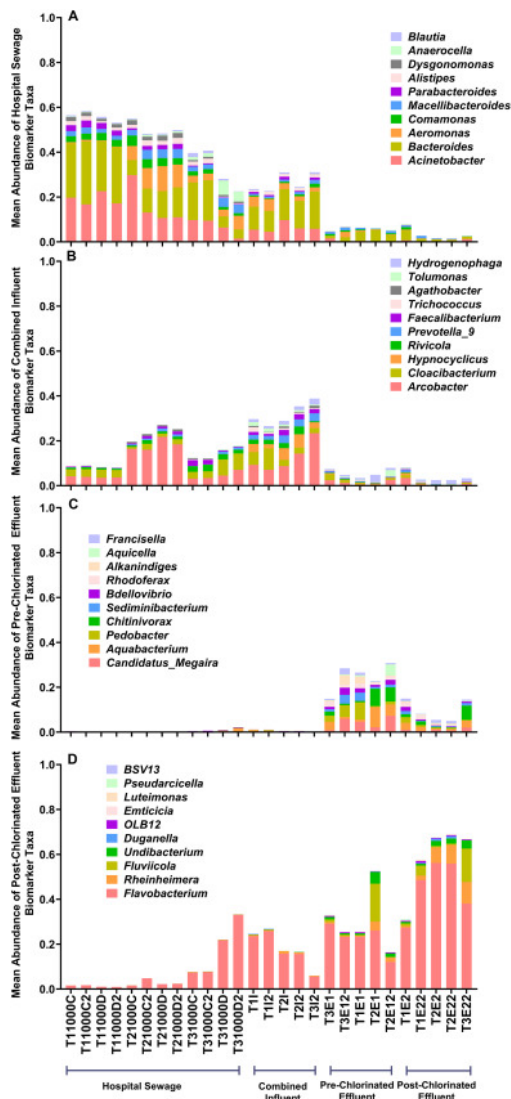


Fig. 2. Abundance of biomarker taxa at the genus level as defined by LEfSe analysis. The relative abundance of biomarker taxa from A) Hospital Effluent, B) Combined Influent, C) Pre-Chlorinated Effluent, and D) Post-Chlorinated Effluent shown for each individual sample. The ten biomarker taxa from each compartment with the highest LDA score are shown.

### 3.2. Hospital sewage microorganisms contribute strongly to Post-Chlorinated Effluent microbial community composition

The microbial community composition of Pre- and Post-Chlorinated Effluent is a result of wastewater treatment and includes both a mixture of bacterial source communities (Hospital Sewage and Combined Influent in this study) and enrichment during the WWTP process. We used FEAST molecular microbial source tracking to determine the potential contribution of different bacterial sources to the final microbial community composition in Pre- and Post-Chlorinated Effluents, separated by sampling date. Across the three sampled time points, twelve Hospital Sewage and five Combined Influent samples served as potential “sources” while five Pre-Chlorinated Effluent and five Post-Chlorinated Effluent samples served as the “sinks”, respectively (Table 2). Hospital Sewage contributes to an average of 5.08% (0.68–9.37%) of the Pre-Chlorinated Effluent microbial community, while Combined Influent contributes to an average of 8.18% (2.38–18.09%) with a peak of 18.90% (Sampling Timepoint 1; Table 2). In contrast, Hospital Sewage contributes to an average of 11.49% (8.89–12.44%) of the Post-Chlorinated Effluent microbial community, while Combined Influent contributes to an average of 4.25% (4.08–5.61%). Although a rather large percentage of microorganisms in the Pre- and Post-Chlorinated Effluent is attributed to “unknown” sources (84.26% average), this result is not unexpected due to other contributing factors of microbial communities within WWTPs including the native WWTP sludge microbial community and enrichment of specific microbial taxa during treatment. We should note that due to our collection timepoints, it is unlikely that these estimated contributions are reflective of microbial populations surviving the WWTP process from Hospital Sewage or Combined Influent to Post-Chlorinated Effluent but rather are reflective of the potential source community due to microbial composition similarity.

Table 2. Contribution of Hospital Effluent and WWTP Combined Influent microbial community composition to WWTP Effluent microbial community composition based on FEAST molecular source tracking results.

Source	Sink	% Contribution <sup>a</sup>	Mean % contribution
Hospital Effluent	Pre-Chlorinated Effluent	1) 0.68 2) 5.17 3) 9.37	5.08
WWTP Combined Influent		1) 18.09 2) 4.07 3) 2.38	8.18
Hospital Effluent	Post-Chlorinated Effluent	1) 12.44 2) 13.13 3) 8.89	11.49
WWTP Combined Influent		1) 4.08 2) 5.61 3) 4.25	4.25

<sup>a</sup>Note that the % contribution is based upon three separate sampling timepoints. Hospital Effluent and Influent samples were used as sources for Pre- and Post- Chlorinated Effluent collected from the same sampling date.

### 3.3. Multiple potential human and animal pathogens are found throughout the wastewater treatment system and in sediments downstream of receiving waters

A primary goal of the wastewater treatment process is pathogen reduction before treated water is released back into the environment to prevent both human and animal disease. We investigated the presence of potential pathogens at the species level using exact sequence matching both throughout the WWTP and within sediment samples from the Milwaukee Inner Harbor and downstream of the JI WWTP as an indicator of potential health risks. A total of 28 potential pathogen species from 21 genera were identified throughout the WWTP process (Table S1). Twelve of the 28 species were found in the highest relative abundance in Hospital

Sewage samples. Two potential pathogen species, *Enterococcus saccharolyticus* and *Elizabethkingia miricola*, were only detectable in Pre-Chlorinated Effluent. While the majority of the 28 identified potential pathogen species were reduced to non-detectable levels in Post-Chlorinated Effluent, five remained including potential human pathogens *Plesiomonas shigelloides*, *Pseudomonas alcaligenes*, *Arcobacter cryaerophilus*, and *Streptococcus equinus*, in addition to the common fish pathogen *Flavobacterium succinicans* (Table S1). *Flavobacterium succinicans* was also present in sediments immediately downstream of the JI outfall in addition to *Chryseobacterium indologenes*, indicating these potential pathogens can survive the entirety of the WWTP process and remain detectable in environmental sediments (see Fig. S1 for sediment sampling locations). Two other potential pathogen species identified in the WWTP process were found in sediments impacted by stormwater outfalls (part of the combined sewage system servicing Milwaukee) including *Arcobacter cryaerophilus* and *Acinetobacter johnsonii*, suggesting they may have also originated within the sewage system. These results suggest chlorination during wastewater treatment does not effectively remove all pathogens. It is likely that both the combined sewer system outfalls and JI outfall contribute to dissemination of multiple potential pathogenic species into the environment posing an environmental and human health threat.

The relative abundance of the seven potential pathogenic species that were identified in Post-Chlorinated Effluent and/or sediments downstream of JI are visualized in Fig. 3. The likely sources of these potential pathogens can be inferred from their relative abundance in the two source samples, Hospital Sewage and Combined Influent, and based on whether the potential pathogen is likely nosocomial or an opportunistic pathogen. Based on these characterizing factors, Hospital Sewage is the most likely source of *Acinetobacter johnsonii* while Combined Influent is the most likely source of *Chryseobacterium indologenes* and *Streptococcus equinus*. Both Hospital Sewage and Combined Influent contribute to the populations of *Arcobacter cryaerophilus*, *Flavobacterium succinicans*, *Plesiomonas shigelloides*, and *Pseudomonas alcaligenes*. Although some of these bacteria are routinely detected in environmental samples, their presence in sediments immediately downstream of the WWTP suggests that the treatment process and the released effluents contribute to their presence of these potential pathogens in the environment. However, the presence of these pathogens could also be attributed to anthropogenic or animal feces contamination as the three rivers that flow through Milwaukee converge near the WWTP outfall site.

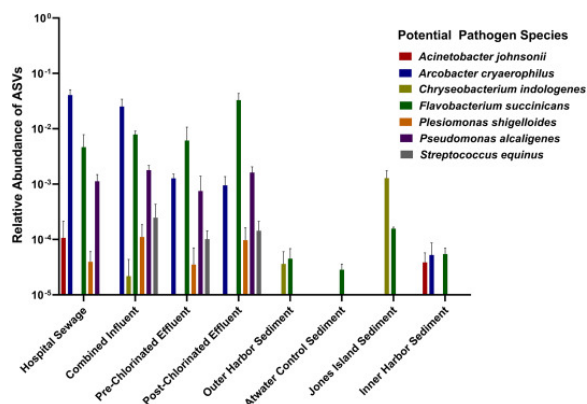


Fig. 3. Relative abundance of potential pathogens identified in Post-Chlorinated Effluent and/or Lake Michigan sediments on a logarithmic scale presented as mean  $\pm$  SD. WWTP compartments are as previously defined. Sediments sampling regions include: Outer Harbor- downstream of the WWTP and outside the Milwaukee Harbor break wall; Atwater- north of the WWTP and outside of the range of impact, Jones Island Sediment- immediately downstream of the WWTP effluent release pipe, and Inner Harbor- meeting location of the three major rivers in Milwaukee, WI which contain water from sewer outflows (see Fig. S1 for reference).

### 3.4. Post-Chlorinated Effluent contains culturable microorganisms resistant to critically important antimicrobials

The dissemination of ARB in the environment is a major concern, and WWTPs have been implicated as both reservoirs and contributors of ARB in surface waters following wastewater treatment. The Centers for Disease Control and Prevention (CDC) has identified resistance to third-generation cephalosporins and carbapenems amongst Enterobacteriaceae as major and urgent threats, respectively (CDC, 2019); thus, we identified the culturable aerobic microbial community composition of Post-Chlorinated Effluent that was resistant to antibiotics of critical importance. We identified the bacterial populations serving as reservoirs for high-risk antibiotic resistance genes using two methods: 1) CFU counts of *Escherichia coli* on mTEC chromogenic agar plates both alone (no antibiotic control) and with ceftazidime (a 3rd generation cephalosporin); and 2) 16S rRNA sequencing of filters plated on TSA media (no antibiotic control) or media containing either ceftazidime or meropenem (a carbapenem). *Escherichia coli* counts remain the standard US-EPA recommended indicator bacteria for fecal contamination of freshwater, but a better understanding of the culturable portion of the antibiotic resistant survivor community following disinfection by chlorination aids in assessing health risks.

The culturable *E. coli* populations decreased throughout the WWTP process from Combined Influent to Post-Chlorinated Effluent by between 4 and 5 logs (Fig. S3-A). *E. coli* were still countable in Post-Chlorinated Effluent; however, the number of total colonies was reduced to <10 total CFUs per 100 mL. The culturable *E. coli* population on plates containing ceftazidime varied significantly between WWTP compartment with the highest recorded resistant *E. coli* found in Combined Influent (Fig. S3-B). Although the resistant *E. coli* population in Pre-Chlorinated Effluent was significantly reduced from the resistant population within Combined Influent, approximately 4.75% of the *E. coli* population in Pre-Chlorinated Effluent remained resistant to ceftazidime (Fig. S3-C). No resistant *E. coli* were detected in Post-Chlorinated Effluent at any timepoint.

The culturable survivor community identified within Post-Chlorinated wastewater using 16S rRNA sequencing was much more diverse than the *E. coli* results alone would suggest (Fig. 4). On filters without antibiotics, the culturable microbial community included a total of twelve genera with high abundances of *Chryseobacterium*, *Aeromonas*, *Bacillus*, and *Acinetobacter*. Of the twelve identified culturable genera, bacteria mapping to seven of those genera were able to survive in the presence of  $\beta$ -lactam antibiotics. Culturable bacteria from filters challenged with ceftazidime were dominated by members of the genera *Acinetobacter*, *Bacillus*, and *Chryseobacterium*, while those challenged with meropenem were predominately mapped to *Chryseobacterium* alone. Sequences recovered from the culturable microbial community challenged with ceftazidime also included multiple ASVs mapped to potential human pathogens at the species level including *Acinetobacter soli*, *Bacillus cereus*, *Bacillus pumilus*, and *Chryseobacterium indologenes*. The diverse antibiotic resistant community surviving in treated wastewater set to be released back into the environment in conjunction with identified antibiotic resistant potential pathogens is concerning for environmental and human health.

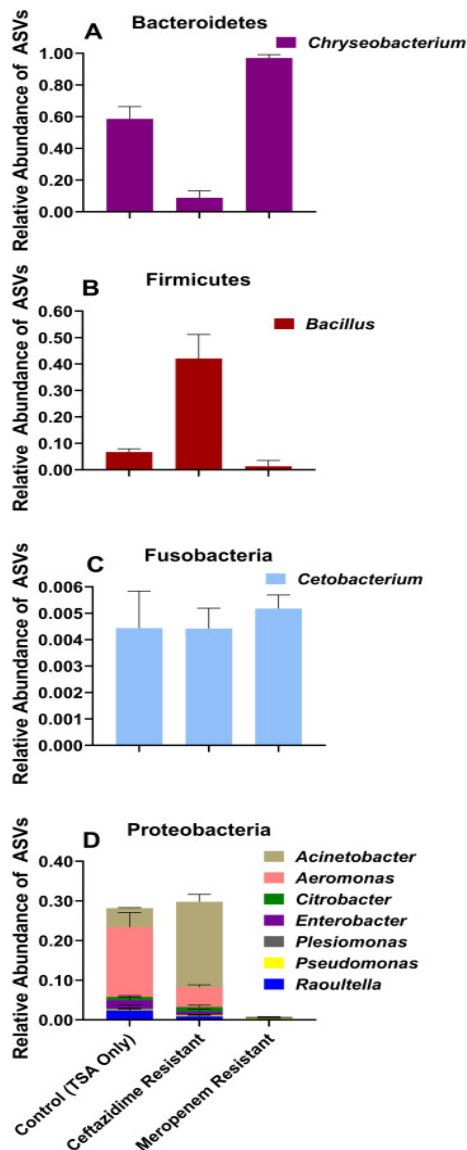


Fig. 4. Relative abundance of amplicon sequence variants (ASVs) from Post-Chlorinated Effluent filters cultured on control (TSA alone) and antibiotic containing media. Genera represented are those with a relative abundance of at least 0.1% across all samples presented as mean  $\pm$  SD. A total of three filters with culturable microbial communities are combined for each sample type.

## 4. Discussion

Municipal WWTPs are the primary form of pollution control for surface waters and are critical for maintaining environmental and public health. Reduction of fecal, pathogenic, and ARB is a crucial function of WWTPs; however, multiple studies have demonstrated the continued presence of these bacterial types in WWTP outflows and receiving waters. In this study, we found that the taxonomic microbial community composition of Hospital Sewage was significantly different than Combined Influent, even though they are both primarily composed of human sanitary waste (Fig. 1). Multiple human gut taxa were significantly more abundant in Hospital Sewage, including the biomarker taxa *Bacteroides*, *Parabacteroides*, and *Ruminococcus*, similar to the findings of Buelow et al. (2018). Additionally, multiple genera known to include human and animal pathogenic species including *Aeromonas*, *Acinetobacter*, and *Elizabethkingia* were significantly more abundant in Hospital Sewage. Members of these genera are frequently associated with hospital acquired infections and have been

isolated from hospital water and disinfectants, increasing the risk of spread (Batra et al., 2016; Joly-Guillou, 2005; Ratnamani and Rao, 2013). Pathogenic *Aeromonas* spp. are particularly dangerous for both human and animal health with infections frequently arising from environmental isolates (Batra et al., 2016) that might exhibit resistance to over 25 different antibiotics (Rizzo et al., 2013), including third-generation cephalosporins and carbapenems (Lan et al., 2017).

A relatively understudied component of WWTPs is the source of microbial populations surviving in disinfected, treated wastewater effluent set to be released back into the environment. Tracking potential pathogens and ARB that survive the WWTP process through to Post-Chlorinated Effluent by using traditional, culture-based microbial source tracking is necessary but remains infeasible. Here, we used molecular microbial source tracking to determine the potential contribution of source microbial communities (Hospital Sewage and Combined Influent) to the microbial community composition of Pre- and Post-Chlorinated Effluents. Although Combined Influent had a higher average estimated contribution in Pre-Chlorinated Effluent, we identified Hospital Sewage as a likely source of microorganisms in Post-Chlorinated Effluent, contributing an estimated 11.49% (Table 2). Due to the timing of sample collection in this study, estimated contributions more strongly reflect microbial composition similarity between source and sink microbial communities, rather than providing a strict identification of microorganisms that survived from Hospital Sewage or Combined Influent through the disinfection process. Additionally, the estimated contribution of Hospital Sewage microbial populations to treated effluent microbial populations should be considered in the context of distance and dilution of these samples between the collection point and the JI WWTP (~12–15 sewer pipe miles). It is possible that microorganisms from uncollected samples within the sewer pipes themselves, storm water, or the pipe biofilm also contribute to the “unknown” portion of the estimated microbial sources but is beyond the scope of this study. Regardless, we believe this result indicates multiple microbial taxa originating in Hospital Sewage (the most upstream microbial source community) are better able to survive chlorination than those originating in Combined Influent and thus are more strongly represented in Post-Chlorinated Effluent. This is supported by the overlap in taxonomy between Hospital Sewage and Post-Chlorinated Effluent which share a total of 270 unique genera in this study. In comparison, Influent and Post-Chlorinated Effluent only share a total of 178 unique genera (data not shown). Documented incidences of hospital associated pathogen resistance to disinfectants, including chlorine, have increased in recent years suggesting these organisms can survive wastewater treatment and disinfection (Abreu et al., 2013; Russell, 1999). Additionally, a recent study found that while chlorination inactivated ARB, it was not effective at reducing erythromycin and tetracycline resistance genes, which suggests horizontal gene transfer could still occur even with efficient bacterial removal (Yuan et al., 2015). However, one alternative possibility for similarities in microbial community composition within Hospital Sewage and Post-Chlorinated Effluent is enrichment due to treatment processes as some of the microorganisms originating in the Hospital Sewage were likely exposed to chlorination disinfection prior to introduction to the sewage system. Culture-based source tracking methods are needed to confirm the origin and survivability of microorganisms from influent sources in disinfected effluents.

This result is concerning as Hospital Sewage can contain an array of antibiotic resistant and pathogenic microorganisms in addition to unmetabolized antibiotics which provide a selective pressure for ARB (Kümmerer et al., 2000). This suggests that a fraction of these microorganisms are capable of surviving the WWTP process and chlorination disinfection and/or are exposed to a selective environment that encourages the proliferation of resistant bacteria. Confirmed antibiotic resistant pathogens including *Klebsiella pneumoniae* and *Proteus mirabilis* from hospital sewage have been isolated in the final sedimentation tank of a wastewater treatment plant (Kalaiselvi et al., 2016). Additionally, the removal efficiency of antibiotics using chlorination disinfection is mixed, with significant removal of erythromycin and trimethoprim, slight removal of sulfamethoxazole, ciprofloxacin, and insignificant removal of tetracycline and norfloxacin (Burch et al., 2019) which can promote the proliferation and dissemination of ARB in Post-Chlorinated Effluent. Although less research has been

conducted to date on the removal efficiency of  $\beta$ -lactams, cephalosporins, and other critically important antibiotics, recent studies have shown a minimal decrease in  $\beta$ -lactam associated ARGs throughout the treatment process (Hiller et al., 2019; Laht et al., 2014; Pazda et al., 2019). In combined sewage systems such as the one studied here, the risk of overflows following heavy precipitation is high, leading to a bypass of treatment altogether and increasing the environmental and human health risks (USEPA, 2004a, USEPA, 2004b). Reducing the source of pathogenic and/or ARB is vital to preventing the reintroduction of these organisms into the environment following wastewater treatment.

In this study, we identified a total of 28 potential human and animal pathogens at the species level throughout the WWTP process, including five potential pathogen species that remained detectable in Post-Chlorinated Effluent (Table S1). The detection of pathogenic bacteria in WWTP effluent is a common occurrence and can comprise as much as 7.6% of the microbial community (Cai and Zhang, 2013). Additionally, we identified four potential pathogenic species in sediments downstream of the WWTP, two of which likely originated in Hospital Sewage (Fig. 3). These results, combined with the elevated levels of pharmaceuticals and personal care products identified by Blair et al. (2013) in the same Lake Michigan sampling locations described in this study, indicate WWTP effluents increase both the abundance of and environmental selective pressure for ARB and pathogens.

Limited data is available on the diversity of ARB populations surviving in disinfected wastewater due to traditional culture-based approaches. Instead, the abundance of culturable *E. coli* is used as a proxy for pathogen load in treated wastewater, and extended spectrum  $\beta$ -lactamase *E. coli* have been suggested as a viable proxy for environmental monitoring of antimicrobial resistance (Ashbolt et al., 2018) However, while we were unable to identify any ceftazidime or meropenem resistant *E. coli* at the species level from Post-Chlorinated Effluent samples with the volumes used for enrichment and sequencing (Fig. S3), we were able to culture a diverse array of potential pathogens exhibiting ceftazidime and meropenem resistance using our combined culture and molecular approach (Fig. 4) indicating that *E. coli* counts are an inefficient proxy for pathogen risk assessment in treated wastewater.

Although ARB have been detected in sewage and receiving waters from studies dating to the 1970s (Linton et al., 1974), a fundamental understanding of sewage and WWTP contributions to environmental ARB is lacking. We identified multiple taxa at the genus and species level known to contain human and animal pathogens including *Acinetobacter*, *Aeromonas*, *Chryseobacterium*, and *Pseudomonas* that were resistant to critical  $\beta$ -lactam antibiotics used in human medicine (ceftazidime and meropenem) (WHO, 2019). Interpreting datasets from 16S rRNA libraries of non-cultured microorganisms can be challenging as they can include nonviable or extracellular DNA sources in addition to the viable organisms of interest. In this study, the culture-based approach combined with 16S rRNA sequencing enabled us to characterize the viable, aerobic subset of the microbial community that is resistant to third-generation cephalosporins and carbapenems, thereby identifying serious and urgent threats within disinfected wastewater. Although data from this study cannot concretely identify the source of resistant populations, Amador et al. suggested meropenem resistance originates in hospital sewage because its use is restricted to clinics (Amador et al., 2015). Korzeniewska and Harnisz (2018) also found  $\beta$ -lactam resistant *E. coli* populations were detected more frequently in wastewater containing hospital sewage. Additionally, a review of the effectiveness of chlorination disinfection practices found mixed results for the removal of ARB, with one study indicating incomplete removal by chlorination resulting in the regrowth of resistant bacteria (Hiller et al., 2019). Together, these results indicate hospital sewage contains a more resilient microbial population which impacts the final microbial community composition of treated effluent thereby jeopardizing receiving freshwaters and increasing risks to human and animal health.

This study did have a few limitations. We focused on the wastewater fraction of the WWTP process; however, activated sludge from JI may also contribute to the microbial community composition of treated effluent. Additionally, our collection of replicate grab samples on three timepoints in October 2018 cannot account for

the entirety of the microbial community in each of the WWTP compartments on a given date or seasonal impacts on the changes in microbial community composition. Lastly, the culture-based method employed here focuses only on bacterial populations with similar doubling times and capable of growing on tryptic soy agar (TSA). Limits to this approach include underrepresentation of slow-growers, absence of fastidious bacterial populations, and an inability to compensate for bacterial competition. Considering these factors, we did not identify antibiotic resistant fastidious or slow-growing bacterial populations. Despite these limitations, we believe the combined culture-based and molecular approach used in this study provide a deeper understanding of the ceftazidime and meropenem resistant microbial community composition of WWTPs. Additionally, this method identified potential carriers of ESBL resistance which may promote horizontal gene transfer in WWTPs, as well as recipient surface water and lake sediment (Laroche-Ajzenberg et al., 2015).

## 5. Conclusions

Wastewater treatment is vital to the health and safety of surface waters used for recreation, drinking water, and animal habitat. In this study, we identified multiple microbial taxa in Hospital Sewage that were also present in treated and disinfected effluents which included potential pathogenic species and  $\beta$ -lactam resistant microbial communities. Four potential pathogen species identified throughout the WWTP were also found within sediments downstream of the WWTP suggesting a subset of potential pathogens can both survive and thrive in the aquatic environment following wastewater treatment. Together, these results suggest that WWTPs receiving hospital sewage, including pretreated hospital sewage as analyzed in this study, are at increased risk for disseminating ARB and pathogens into the environment, putting human and environmental health at risk. One possible mitigation strategy to reduce the number of microorganisms released from Hospital Sewage into the primary sewer system and downstream receiving waters is to include full-scale, on-site wastewater treatment at the hospital prior to release into the municipal sewage system (Barancheshme and Munir, 2018; Korzeniewska et al., 2013). Wastewater treatment processes do significantly reduce microbial loads and ARBs, although their continual presence in both treated wastewater and downstream sediment suggest additional treatment beyond chlorination disinfection, as well as better tracking systems, are needed to reduce dissemination and proliferation of pathogens and ARB.

## CRedit authorship contribution statement

**Rachelle E. Beattie:**Data curation, Investigation, Formal analysis, Methodology, Visualization, Writing - original draft, Writing - review & editing.**Troy Skwor:**Data curation, Funding acquisition, Investigation, Methodology, Resources, Writing - review & editing.**Krassimira R. Hristova:**Conceptualization, Funding acquisition, Resources, Supervision, Writing - review & editing.

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## Data availability

Sequencing files are available in the National Center for Biotechnology Information Sequence Read Archive under BioProject ID Number PRJNA622864.

## Declaration of competing interest

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

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