"Does Increasing Solids Retention Time in the Wastewater Treatment Process Affect the Persistence of Antibiotic Resistance Genes?"

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Problem and Research Objectives:

A 2000 World Health Organization report focused on antibiotic resistance (AR) as one of the most critical human health challenges of the next century and heralded the need for "a global strategy to contain resistance" **[1]**. According to the report, more than 2 million Americans are infected each year with resistant pathogens, and 14,000 die as a result. Following their use, it is estimated that up to 75% of antibiotics are excreted unaltered or as metabolites **[2]**. Unfortunately, most wastewater treatment plants (WWTPs) are not designed for the removal of these micro-pollutants, and as a result, residual antibiotics are released into the environment with treated wastewater, leading to concern regarding their contribution to AR in environmental microorganisms **[3]**. There also exists the potential for wastewater treatment (WWT) processes to select for the survival of resistant microorganisms. Thus, it has been proposed that resistance development during WWT is an important and key source of AR in the environment **[4]**. And yet, few studies have attempted to identify processes contributing to the selection of AR bacteria. Such information will be critical in the development of WWT strategies to reduce environmental transfer of AR bacteria.

During the conventional activated sludge (CAS) step of WWT, the wastewater containing organic matter is aerated in a basin in which micro-organisms metabolize the suspended and soluble organic matter. Because CAS, by its very design, exposes bacteria to ideal growth conditions and relatively high concentrations of antibiotics, it is hypothesized that CAS may increase AR development. Direct correlations between solids retention time (SRT) and reductions in antibiotics have been shown **[5, 6]**, but higher SRTs also provide prolonged exposure of bacteria to influent antibiotic levels. This study proposed to assess the effects of varying SRT in full-scale activated sludge processes on the degradation of trace antibiotics and microbial selection for AR. As the adoption of recycled water (including Indirect Potable Reuse) becomes more widespread, and as the public comes into contact with recycled water at a higher frequency, there will be increased pressure for utilities and other water managers to better understand the microbial population dynamics. Of critical importance will be an improved understanding of microbial populations that could pose a risk to the public. Standardized qualitative and quantitative methods must be developed to better understand risk. A detailed assessment of rates in AR development and identification of bacterial processes contributing to AR will aid in technological advances to decrease the prevalence of AR in recycled water, alleviating environmental and public health concerns.

This study included a comprehensive evaluation of temporal variability in loadings of antibiotic concentrations in the WWT process, quantification of genes conferring AR to bacteria, and examination of relative proportions of AR *E. coli* (Gram negative) and *Enterococcus* (Gram positive) in raw wastewater, activated sludge solids, and finished effluent from a range of

treatment facilities. The primary goal of this research was to focus on operational conditions during biological treatment, since these processes may pose the greatest risk for the development of AR populations. By monitoring several locations within the WWT train, project team was able to characterize the impact of WWT on AR prevalence and, in turn, to depict the downstream impacts of recycled water on end-users and the environment. Ultimately, this study will provide utilities with new knowledge and tools for treatment process optimization and AR mitigation.

Methodology:

Task 1: Literature Review. The first task involved a review of available literature related to AR in water supplies, supplemented with a review of occurrence and usage patterns for widely used prescription pharmaceuticals, including human metabolism rates, and susceptibility to common WWT processes. Five target antibiotics (sulfamethoxazole, trimethoprim, ampicillin, tetracycline, vancomycin) and their associated quantitative analytical methods (described below) were finalized during this task. This task concluded with the selection of the quantitative PCR (qPCR) assays for enumeration of select bacterial genes conferring resistance to the target antibiotics (Table 1).

Table 1. qPCR Assays for Antibiotic Resistance Gene Analysis

Primers	Assay Target	Sequences	Amplicon size (bp)	References
*sul*I-F *sul*I-R	Sulfamethoxazole	cgcaccggaaacatcgctgcac tgaagttccgccgcaaggctcg	163	Pei et al., 2006
*sul*II-F *sul*II-R	Sulfamethoxazole	tccggtggaggccggtatctgg cgggaatgccatctgcctgag	191	Pei et al., 2006
dfr1-F dfr1-R	Trimethoprim	cgaagaatggagttatcggg tgctggggatttcaggaaag	372	Grape, M., 2007
Lak2-F Lak1-R	Ampicillin	gggaatgctggatgcacaa catgacccagttcgccatatc	189	Volkmann et al., 2003
*tet*W-F *tet*W-R	Tetracycline	gagagcctgctatatgccagc gggcgtatccacaatgttaac	168	Aminov et al., 2001
vana3-F vana3-R	Vancomycin	ctgtgaggtcggttgtgcg tttggtccacctcgcca	377	Volkmann et al., 2003; Merlino et al., 2010
GFD-F GFD-R	Helicobacter spp.	ctatgacgggtatccggc attccacctacctctccca	376	Proietti et al., 2010; Green et al., 2011
Bac-F Bac-R	Bacteria 16s rRNA	atggttgtcgtcagct acgggcggtgtgtac	370	Ritalahti et al., 2006

Task 2: Full-Scale Sampling to Quantify Antibiotic and AR Loadings. During previous research projects (WERF-CEC4R08, WRF-08-05, and WRF-09-10), the project team developed collaborative relationships with WWTPs throughout Arizona and the U.S. These existing collaborations provided a foundation for this study, and eight facilities were selected based on their range in operational conditions specifically related to SRT (1.5 to 25 days). Samples were

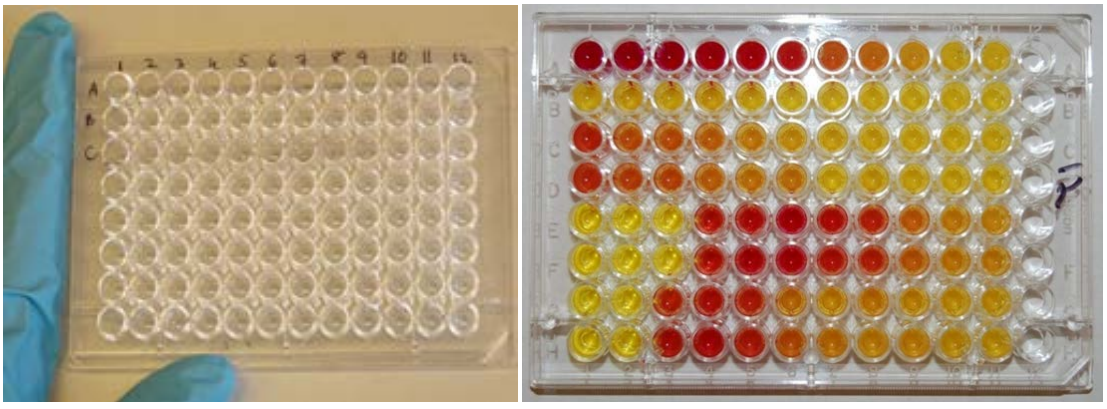
collected at two locations within each WWTP. For the microbial analyses described below, samples were collected from the primary clarifier and the discharge from the activated sludge basin (immediately prior to the secondary clarifiers). The suite of antibiotics finalized in Task 1 was quantified in the primary and secondary effluent.

Task 3: Analysis of Full-Scale Sampling Data. During Task 1, the treatment trains included in this study were characterized based on unit processes and operational conditions. Although the entire treatment train was characterized, we focused on conditions associated with CAS since this process may provide the greatest potential for the development of AR. The conditions encompassed by the selected facilities allowed the project team to identify the operational parameter(s) with greatest impact on AR prevalence. This was accomplished by evaluating correlations between each operational variable (e.g., SRT, type of biological treatment) and the relative concentrations of AR microbes and genes.

Antibiotic concentrations were analyzed using automated solid phase extraction (Dionex), isotope dilution, liquid chromatography (Aquity UPLC, Waters), and tandem mass spectrometry (MS/MS; Quattro Premier XE, Waters) in the Arizona Laboratory for Emerging Contaminants (ALEC) at the University of Arizona. Studies have shown that trace concentrations of antibiotic compounds in treated effluent are significantly lower than the antibiotic concentrations commonly used for resistance evaluation. For the microbial samples, *E. coli* and *Enterococcus* were selectively enriched and isolated on agar plates.

Individual *E. coli* and *Enterococcus* isolates were added to 96-well plates serially diluted with target antibiotics (Fig. 1). Following a 24-h incubation period, sample absorbance at 600 nm, which is indicative of microbial growth, was quantified for each well. According to CLSI standards **[10]**, the minimum inhibitory concentration (MIC), or the lowest antibiotic concentration that inhibits visible growth, was reported for each isolate. According to our hypothesis, isolates collected from facilities with higher SRTs should be characterized by higher MICs.

Figure 1. *The image below shows a 96-well plate experimental set up. A single isolate in growth medium was added to all cells in columns A and B, while column C received medium only as negative growth control. Rows 2-8 were pre-loaded with antibiotics. Row 1 = positive growth control wells; no antibiotic. Row 2 = lowest level of antibiotic; Row 3 = 2X antibiotic concentration of Row 2; Row 4 = 2X antibiotic concentration of Row 3…Row 8 = highest concentration.*



Finally, DNA was extracted in triplicate from each raw sample collected through the WWT train. Real-time qPCR was used to quantify genes within the DNA encoding resistance to target antibiotics. Internal control DNA from Helicobacter spp. (GFD; Table 1) was spiked into each sample prior to DNA extraction to quantify DNA extraction efficiency. Finally, conserved portions of the Universal 16Sr RNA gene were quantified within each sample (Table 1) to standardize PCR results and allow for direct comparison between samples. Our hypothesis for this Task was that AR genes would be expressed with higher frequency in samples collected from facilities with higher SRTs.

Principal Findings and Significance:

Results from this study suggest that while prolonged SRTs may be beneficial at reducing residual levels of trace organic contaminants they also may prolong the exposure of native microbial populations to antibiotics and thus confer antibiotic resistance. In this study we evaluated eight wastewater treatment facilities with SRTs ranging from 1 to 25 days (Table 2.)

It is anticipated that results of this work could permit optimization of SRT at each facility for the enhanced degradation of Trace Organic Contaminants as well as reduction in Antibiotic Resistant microorganisms.

Table 2. Wastewater Treatment Plant Operational Parameters. Treatments included; Trickling Filter (TF); Conventional Activated Sludge (CAS); Chlorination (Cl); Ultraviolet Light (UV); Membrane Bioreactor (MBR); and Sequencing Batch Reactor (SBR).

WWTP Site	SRT (days)	BOD (mg/L)	MGD	Treatment
Plant 1	1-2	243	35	TF
Plant 2	2-4	253	9	CAS/Cl/UV
Plant 3	4	263	8	MBR
Plant 4	8-9	167	9	CAS/Cl/UV
Plant 5	14	210	2	CAS/Cl/UV
Plant 6	17	245	10	CAS/Cl/UV
Plant 7	19	328	135	CAS/Cl/UV
Plant 8	25	282	2	SBR

The following tables (Tables 4 – 8) represent the percentage of bacterial isolates classified as “resistant” as defined by the Clinical and Laboratory Standards Institute (CLSI). CLSI updates and standardizes MIC levels at which bacteria are considered “resistant” (Table 3). Individual

isolates were screened against a range in concentrations of antibiotics that bracketed the CLSI standards.

Table 3. Clinical and Laboratory Standards Institute (CLSI) Standards for Target Resistance

Antibiotic	Concentration Range Tested (µg/ml)	Target Resistance (µg/ml)
Tetracycline	2-128	≥16
Sulfamethoxazole	8-512	≥64
Trimethoprim	2-128	≥16
Ampicillin	2-128	≥32
Vancomycin	0.5-32	≥4

Table 4. Vancomycin % Isolates Tested that Displayed High Level Resistance

Vancomycin	Primary Treatment	Secondary Treatment
SRT of 3 days	95%	63%
SRT of 9 days	95%	90%
SRT of 19 days	95%	83%

Table 5. Sulfamethoxazole % Isolates Tested that Displayed High Level Resistance

Sulfamethoxazole	Primary Treatment	Secondary Treatment
SRT of 3 days	29%	0%
SRT of 9 days	37%	37%
SRT of 19 days	8%	29%

Table 6. Ampicillin % Isolates Tested that Displayed High Level Resistance

Ampicillin	Primary Treatment	Secondary Treatment
SRT of 3 days	58%	0%
SRT of 9 days	45%	37%
SRT of 19 days	95%	75%

Table 7. Trimethoprim % Isolates Tested that Displayed High Level Resistance

Trimethoprim	Primary Treatment	Secondary Treatment
SRT of 3 days	75%	33%
SRT of 9 days	45%	20%
SRT of 19 days	75%	75%

Table 7. Tetracycline % Isolates Tested that Displayed High Level Resistance

Tetracycline	Primary Treatment	Secondary Treatment
SRT of 3 days	95%	45%
SRT of 9 days	75%	45%
SRT of 19 days	95%	75%

For each of the five antibiotics evaluated across the range of SRTs, a general trend of decreasing percent resistance in effluent collected from the primary treatment to samples collected from the secondary clarifier. This indicates that the treatment process at each of the facilities is effective at reducing some level of resistance in the bacterial populations. However, when evaluating the total percent resistance after secondary treatment, facilities with SRTs of 3 days ranged from 0% to 63% resistance while SRTs of 19 days had substantially higher levels of resistance ranging from 29% to 83%. This result supports the hypothesis that increasing SRT aids the persistence and development of antibiotic resistant bacterial populations.

An additional way of interpreting the development of antibiotic resistance is to measure the Minimum Inhibitory Concentration (50) or MIC_{50}. While high level resistance (Tables 4-8) indicates results based on single isolates, MIC_{50} represents resistance in a large group or organisms. MIC_{50} is defined as the antibiotic concentration required to inhibit the growth of 50% of organisms within a bacterial population. Tables 9-13 represent the MIC_{50} for low (3 days), midrange (9 days), and high (19 days) SRTs for each of the 5 antibiotics evaluated.

Table 9.Vancoymycin MIC_{50}

Vancomycin	Primary Treatment	Secondary Treatment
SRT of 3 days	32 µg/ml	16 µg/ml
SRT of 9 days	8 µg/ml	32 µg/ml
SRT of 19 days	8 µg/ml	32 µg/ml

Table 10. Sulfamethoxazole MIC_{50}

Sulfamethoxazole	Primary Treatment	Secondary Treatment
SRT of 3 days	32 µg/ml	8 µg/ml
SRT of 9 days	16 µg/ml	32 µg/ml
SRT of 19 days	16 µg/ml	32 µg/ml

Table 11. Ampicillin MIC_{50}

Ampicillin	Primary Treatment	Secondary Treatment
SRT of 3 days	64 µg/ml	32 µg/ml
SRT of 9 days	64 µg/ml	64 µg/ml
SRT of 19 days	64 µg/ml	128 µg/ml

Table 12. Trimethoprim MIC_{50}

Trimethoprim	Primary Treatment	Secondary Treatment
SRT of 3 days	32 µg/ml	8 µg/ml
SRT of 9 days	64 µg/ml	32 µg/ml
SRT of 19 days	64 µg/ml	32 µg/ml

Table 13. Tetracycline MIC_{50}

Tetracycline	Primary Treatment	Secondary Treatment
SRT of 3 days	128 µg/ml	64 µg/ml
SRT of 9 days	32 µg/ml	32 µg/ml
SRT of 19 days	64 µg/ml	128 µg/ml

Results from the MIC_{50} analysis agree with results from the percent resistance analysis in that SRTs of 3 days show a decrease in the concentration of antibiotic needed to inhibit 50 percent of the bacterial population from the primary clarifiers to secondary treatment. This result was seen for all five antibiotics evaluated. Additionally, four out of the 5 antibiotics evaluated revealed, increases in the MIC_{50} for SRTs of 19 days suggesting that increasing SRT induces resistance to each individual antibiotic and thus a higher concentration of antibiotic is required to inhibit growth of 50% of the bacterial isolates evaluated.

Summary of Conclusions:

- Results indicate the presence of all target resistance genes (Table 1) from the five antibiotics evaluated along the treatment train of each facility tested.

- Quantitative data indicate that antibiotic resistance genes are decreasing along the treatment train; however, target genes are still found at detectable levels towards the end of treatment.

- Normalized numbers of copies of Bacterial 16S rRNA genes were similar through treatment indicating that while bacteria community composition may change during treatment, total bacterial population concentrations remain essentially unchanged through the treatment process.

- All wastewater treatment plants evaluated were effective at lowering the percentage of resistant bacterial isolates from primary to secondary treatment indicating the success of the treatment regimes in Arizona.

- Solid Retention Times ranging from 1 to 6 days appeared to be the most effective at mitigating antibiotic resistance when compared to SRTs of 9 to 25 days.

- Approximately 35% of isolates showed multiple drug resistance (MDR) indicating resistance to at least 2 antibiotic compounds evaluated.

- Multiple variables within wastewater treatment outside of CAS should be investigated further to better understand the true impact of wastewater treatment on trace organics and their impact on microbial populations. Including: heavy metals, anoxic zones, the de-nitrification processes, disinfection processes, etc.

- Future investigation should include tertiary treatment and evaluate the presence of antibiotic resistant bacterial isolates and resistance genes in the final effluent. In addition, future work must evaluate the impact of resistance bacteria and genes on development of

biofilms within water transport systems and on native bacterial populations in the environment.

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