Solids Retention Time, Influent Antibiotic Concentrations, and Temperature as Selective Pressures for Antibiotic Resistance in Activated Sludge Systems

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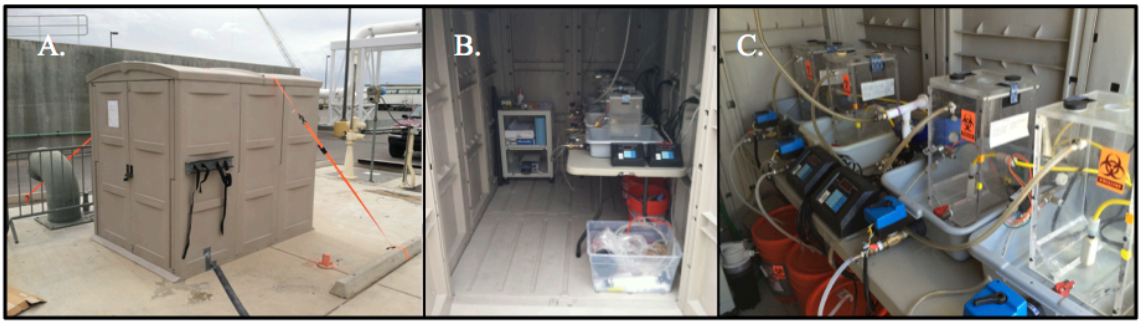
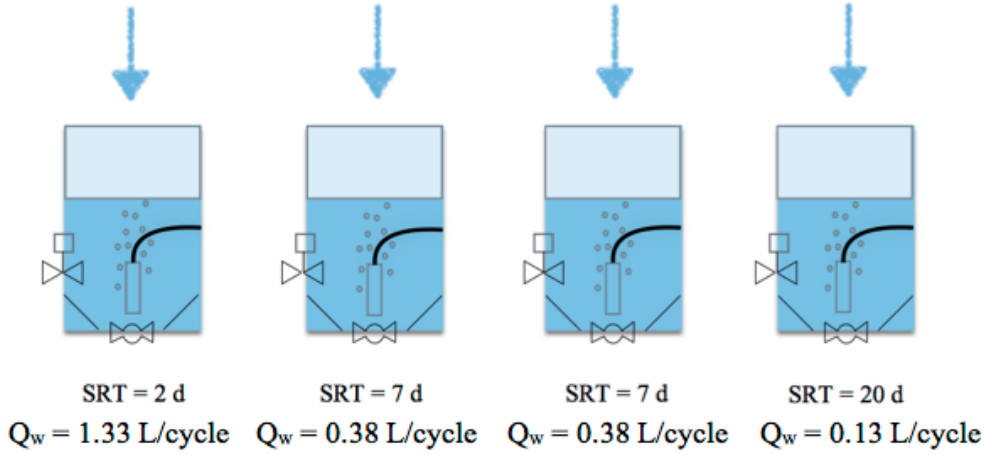
Figure S1. *Top:* Photos of the (A) exterior of the experimental shed, (B) interior of the shed, and (C) parallel sequencing batch reactors (SBRs). The reactors were set up at a full-scale municipal wastewater treatment plant in Las Vegas, NV. The reactors were fed with primary effluent from the full-scale facility. *Bottom:* Schematic of the SBRs, target solids retention times (SRTs), and target waste activated sludge (WAS) flow rates (Q_w).





Supporting text: A MasterFlex peristaltic pump (Model 77200-62, Cole Parmer, Vernon Hills, IL) was used to transfer primary effluent from a wet well through a polytetrafluoroethylene/stainless steel strainer (Hach, Loveland, CO) and a 50-µm cartridge filter (Watts WPC50-975) prior to filling the reactors. The cartridge filters were replaced every two days to mitigate fouling and anaerobic conditions. A four-station irrigation timer (Orbit, Bountiful, UT) was used to control the volume fed to each reactor. Electric actuated solenoid valves (Parker Hannifin Corporation, Cleveland, OH) and an industrial grade air compressor (Porter-Cable PCFP02003; 3.5 gallons; 135 psi) were used to aerate the SBRs to achieve a relatively constant dissolved oxygen concentration of 3 to 4 mg/L. The compressed air was passed through a pressure gauge and air flow meter before being fed into the SBRs via stone diffusers. Aeration was sufficient to achieve adequate mixing of the mixed liquor without the need for mechanical mixing. The target SRTs were achieved by wasting predetermined volumes of mixed liquor with four electric actuated ball valves (W.E. Anderson, Michigan City, IN).

The SBRs were operated with a cycle time of 8 hours for 3 cycles per day. Each cycle consisted of the following five stages: (1) filling with primary effluent for 29 minutes as the irrigation timer cycled through each reactor, (2) immediate aeration for 6.5 hours, (3) solids settling for 1 hour, (4) discharge of settled effluent for 30 minutes, and (5) idle for 1 minute. Solids wasting was performed toward the end of each aeration phase to minimize clogging of the ball valves. The target WAS flow rates (Q_w) were determined according to Eq. S1.

$$Q_w = \left(\frac{1}{f}\right)\left(\frac{V_R}{\theta_c} - \frac{Q_e * C_e}{C}\right) = \frac{V_R}{f * \theta_C} \text{ (when } C_e \approx 0) \qquad \text{(Eq. S1)}$$

where, Q_w = WAS flow rate, L/cycle
f = frequency, cycles/day
V_R = volume of the SBR, L
θ_C = target SRT, days
Q_e = effluent flow rate, L/day
C_e = total suspended solids in settled effluent, mg/L
C = mixed liquor suspended solids, mg/L.

SBR testing was divided into two phases, as summarized below:

Phase 1 – Solids Retention Time

- Duration = ~60 days
- Temperature decreased from 30°C to 10°C
- Antibiotic concentrations = 1x (four reactors)
- Solids retention times = primary effluent, 2 days, 7 days (two reactors), 20 days
- Antibiotic resistance testing = 3 sample events
    - AMP/SMX/TMP Resistance: 3, 31, 55 days post-startup
    - VA/TET Resistance: 5, 33, 53 days post-startup
- Trace organic compound testing = 2 sample events
    - 57 and 58 days post-startup

Phase 2 – Influent Antibiotic Concentrations

- Duration = ~60 days
- Temperature increased from 15°C to 25°C
- Antibiotic concentrations = 1x, 10x (two reactors), 100x
- Solids retention times = primary effluent, 7 days (four reactors)
- Antibiotic resistance testing = 3 sample events
    - AMP/SMX/TMP resistance: 3, 31, 14* days post-startup
    - VA/TET resistance: 5, 33, 16* days post-startup
- Trace organic compound testing = 2 sample events
    - 16* and 17* days post-startup
- *The reactors were restarted after ~45 days of operation during phase 2 due to an unforeseen scheduling issue. The SBRs were operated for an additional 14/16 days prior to the third sampling event to allow for steady state conditions.

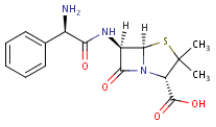
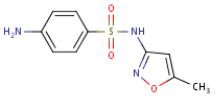
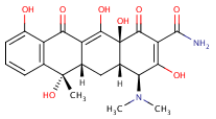
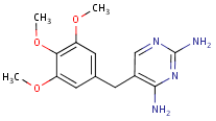
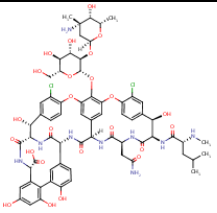
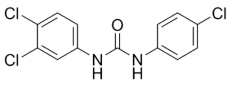
Figure S2. *Top:* Photos of (A) timers and valves used to control flow of primary and secondary effluent, compressed air, and WAS and (B) an example of one of the SBRs at the end of the settling phase/start of the discharge phase. *Bottom:* Photos of (C) system used to deliver antibiotic spike solutions and (D) SBR configuration during phase 2 testing.

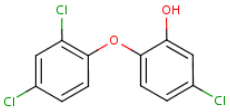
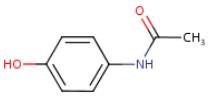
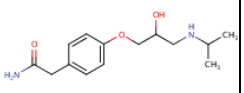
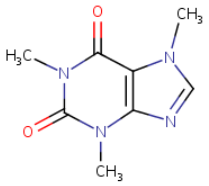
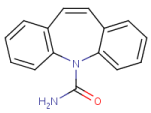
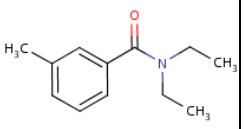
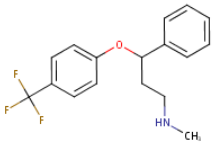
Supporting text: The target antibiotic concentrations were achieved with an automated feed system consisting of styrofoam coolers containing reactor-specific antibiotic stock solutions and small peristaltic pumps (12V DC) connected to a timer. The concentrated stock solutions were prepared so they would achieve the target antibiotic concentrations after dilution in the reactors. The stock solutions were stored on ice in the coolers and replaced every two days to minimize target compound degradation.

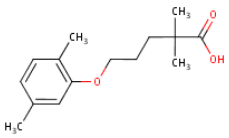
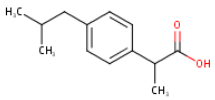
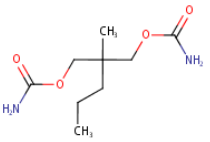
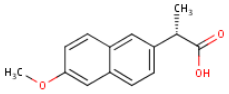
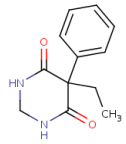
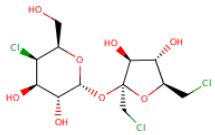
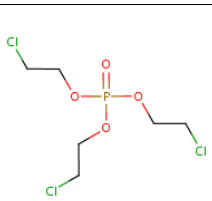
Table S1. Summary of methods for water quality parameters.

Measurement	Sampling and Measurement Method	Analysis Method	Sample Container/ Quantity of Sample	Preservation/ Storage	Hold Time
pH	Orion Model 720A pH meter	Standard Method 4500-H B	20 mL glass vials/10 mL	None	Immediate analysis
MLSS	0.45-µm glass fiber filters, 25-mL baking crucibles, 105ºC oven, analytical balance	Standard Methods 2540 D	50 mL centrifuge tube/10 mL	Refrigeration/ Store @ 4±2°C	7 d
MLVSS	0.45-µm glass fiber filters, 25-mL baking crucibles, 550ºC oven, analytical balance	Standard Methods 2540 D,E	50 mL centrifuge tube/10 mL	Refrigeration/ Store @ 4±2°C	7 d
NH_3	Hach DR/5000 spectrophotometer, Salicylate Method	Hach Method 10031	150 mL amber glass bottle/100 µL	HCl addition to pH<2 / Store @ 4±2°C	28 d
NO_3	Hach DR/5000 spectrophotometer, Cadmium Reduction Method	Hach Method 8039	150 mL amber glass bottle/10 mL	Filter / Store @ 4±2°C	48 h
NO_2	Hach DR/5000 spectrophotometer, Diazotization Method	Hach Method 8507	150 mL amber glass bottle/10 mL	Filter / Store @ 4±2°C	48 h
DO	O_2 electrode probe	Standard Method 4500-O G	40 mL glass vials/20 mL	None	Immediate analysis
sCOD	Hach DR/5000 spectrophotometer, Reactor Digestion Method	U.S. EPA method 410.4, Hach Method 8000	20 mL glass vials/2 mL	H_2SO_4 addition to pH<2 / Store @ 4±2°C	28 d
Chromium (VI)	Hach DR/5000 spectrophotometer, Alkaline Hypobromite Oxidation Method	Hach Method 8023	150 mL amber glass bottle/10 mL	HNO_3 addition to pH<2 / Store @ 20±2°C	6 m
Copper	Hach DR/5000 spectrophotometer, Bicinchoninate Method	Hach Method 8506	150 mL amber glass bottle/10 mL	HNO_3 addition to pH<2 / Store @ 20±2°C	6 m
Zinc	Hach DR/5000 spectrophotometer, Zincon Method	Hach Method 8009	150 mL amber glass bottle/10 mL	HNO_3 addition to pH<2 / Store @ 20±2°C	6 m

Table S2. Summary of target antibiotics and indicator TOrCs.

Compound	Structure[a]	Molecular Formula	Molecular Mass	Biodegradation Potential[b,c]	Sorption Potential[b,c]
Antibiotic TOrCs					
Ampicillin		$C_{16}H_{19}N_3O_4S$	349.41	Low[d]	High[d]
Sulfamethoxazole		$C_{10}H_{11}N_3O_3S$	253.28	Low/Moderate	Moderate
Tetracycline		$C_{22}H_{24}N_2O_8$	444.44	Low[d]	High[d]
Trimethoprim		$C_{14}H_{18}N_4O_3$	290.32	Moderate/High	Moderate
Vancomycin		$C_{66}H_{75}Cl_2N_9O_{24}$	1449.26	Low[e]	High[e]
Antimicrobial TOrCs					
Triclocarban		$C_{13}H_9Cl_3N_2O$	315.58	Low	High

Triclosan		$C_{12}H_7Cl_3O_2$	289.54	High	High
Indicator TOrCs					
Acetaminophen		$C_8H_9NO_2$	151.16	High	Low
Atenolol		$C_{14}H_{22}N_2O_3$	266.34	Moderate	Moderate
Caffeine		$C_8H_{10}N_4O_2$	194.19	High	Low
Carbamazepine		$C_{15}H_{12}N_2O$	236.27	Low	Low
DEET		$C_{12}H_{17}NO$	191.27	Moderate	Low
Fluoxetine		$C_{17}H_{18}F_3NO$	309.33	Moderate	High

Gemfibrozil		$C_{15}H_{22}O_3$	250.34	Moderate	Moderate
Ibuprofen		$C_{13}H_{18}O_2$	206.29	High	Moderate
Meprobamate		$C_9H_{18}N_2O_4$	218.25	Low/Moderate	Moderate
Naproxen		$C_{14}H_{14}O_3$	230.26	High	Moderate
Primidone		$C_{12}H_{14}N_2O_2$	218.25	Low	Low
Sucralose		$C_{12}H_{19}Cl_3O_8$	397.64	Low	Low
TCEP		$C_6H_{12}Cl_3O_4P$	285.48	Low	Low

[a]http://toxnet.nlm.nih.gov; [b]Salveson et al. (2012); [c]Gerrity et al. (2013); [d]Li and Zhang (2010); [e]Current study

Table S3. Summary of analytical methods for target compounds.

Compound (Manufacturer)	Isotope Dilution Standard (Manufacturer)	Precursor Ion	Product Ion	MRL[1] (ng/L)
Positive Electrospray Ionization				
Atenolol (ERA)	Atenolol-d_7 (CDN)	267	145	1.0
Caffeine (ERA)	Caffeine- d_9 (CDN)	195	138	5.0
Carbamazepine (ERA)	Carbamazepine-d_{10} (CDN)	237	165	0.50
DEET (ERA)	DEET- d_6 (CDN)	192	119	1.0
Fluoxetine (ERA)	Fluoxetine-d_5 (CDN)	310	44	0.50
Meprobamate (ERA)	Meprobamate-d_3 (TRC)	219	158	0.25
Primidone (ERA)	Primidone- d_5 (CDN)	219	162	0.50
Sulfamethoxazole (ERA)	Sulfamethoxazole-d_4 (TRC)	254	156	0.25
TCEP (ERA)	TCEP-d_{12} (Isotec)	285	99	10
Tetracycline (Sigma)	Tetracycline-d_6 (TRC)	455	410	5.0
Trimethoprim (ERA)	Trimethoprim-d_9 (TRC)	291	261	0.25
Vancomycin (Sigma)	Vancomycin-d_{12}(ALSACHIM)	725	144	1.0
Negative Electrospray Ionization				
Acetaminophen (ERA)	Acetaminophen-d_4 (TRC)	150	107	0.50
Ampicillin (Fluka)	Ampicillin-d_5 (TRC)	384	207	5.0
Gemfibrozil (ERA)	Gemfibrozil-d_6 (TRC)	249	121	0.25
Ibuprofen (ERA)	Ibuprofen- d_3 (CDN)	205	161	1.0
Naproxen (ERA)	Naproxen-d_3 (CDN)	229	169	0.50
Sucralose (ERA)	Sucralose-d_6 (CDN)	395	35	25
Triclocarban (ERA)	Triclocarban-d_4 (CDN)	313	160	2.0
Triclosan (ERA)	Triclosan-d_3 (CDN)	287	35	1.0

[1]MRLs higher for some samples (e.g., primary effluent) due to matrix effects and/or dilution (indicated in tables)

Supporting text:

Standards and reagents: Certified standard solutions for each target compound were purchased commercially along with corresponding isotopically-labeled versions (Table S3). Trace analysis grade methanol was obtained from Burdick and Jackson (Muskegon, MI). Methyl-t-butyl ether (MTBE) was purchased from EM Science (Gibbstown, NJ), ammonium acetate was purchased from J.T. Baker (Phillipsburg, NJ), and formic acid was purchased from Thermo Scientific (Rockford, IL). Stock solutions and calibrations were prepared in 70% methanol/water (v/v) and stored at -4^{o}C until use.

Solid phase extraction: Solid phase extraction (SPE) protocols were based on work by Vanderford and Snyder (2006). Analytes were extracted from aqueous samples in batches of six using 6-mL, 200-mg hydrophilic-lipophilic balance (HLB) cartridges from Waters Corporation (Millford, MA). Extractions were performed on an AutoTrace™ automated SPE system (Dionex Corporation, Sunnyvale, CA). The SPE cartridges were sequentially preconditioned with 5 mL of MTBE, 5 mL of methanol, and 5 mL of reagent water. As dictated by sample matrix and projected analyte concentration levels, sample aliquots of either 500 mL or 25 mL were spiked

with a solution of isotopically-labeled standards that contained a stable isotope of each analyte, then loaded onto the cartridges at 15 mL/min. Cartridges were then rinsed with 5 mL of reagent water and subsequently dried under a nitrogen stream for 30 min. Each cartridge was then eluted with 5 mL methanol followed by 5 mL of 10/90 (v/v) methanol/MTBE, and both fractions collected in a single 15 mL calibrated centrifuge tube. The resulting extract was concentrated with a gentle stream of nitrogen to volume just below 500 µL, then brought to a final volume of 500 µL using methanol.

Instrumental analysis: Vancomycin and tetracycline were separated using an Agilent (Palo Alto, CA) G1312A binary pump and an HTC-PAL autosampler (CTC Analytics, Zwingen, Switzerland). Previously published separation methods were modified and validated in-house for the analysis of vancomycin (Zhang et al., 2014) and tetracycline (Lopes et al., 2011). Analytes were separated using a 100×4.6-mm Synergi 4-µm polar-RP column (Phenomenex, Torrance, CA). Chromatographic separation was accomplished using a binary gradient of 0.1% formic acid (v/v) in water (A) and 100% methanol (B) at a flow rate of 800 µL/min. The gradient range was 10% B to 100% over 10 min, with a 1-min equilibration step at 10% B. An injection volume of 10 µL was used for analyses.

All other target analytes were separated using an Agilent (Palo Alto, CA) G1312A binary pump and an HTC-PAL autosampler (CTC Analytics, Zwingen, Switzerland) equipped with a 100×4.6-mm Onyx monolithic C18 column (Phenomenex, Torrance, CA). Chromatographic separation was accomplished using a binary gradient of 5 mM ammonium acetate (v/v) in water (A) and 100% methanol (B) at a flow rate of 800 µL/min. The gradient range was 10% B to 100% over 7 min, with a 2-min equilibration step at 10% B. An injection volume of 5 µL was used for analyses.

LC-MS/MS was performed using an API 4000 triple-quadrupole mass spectrometer (SCIEX, Redwood City, CA). The process of optimization of the mass spectrometer has been previously published (Vanderford et al., 2003). Briefly, analytes were grouped into negative electrospray ionization (ESI) or positive ESI based on sensitivity and selectivity for each compound. Once this was established, the optimal compound-dependent parameters were determined and source-dependent parameters optimized for each compound group. Data were collected in scheduled MRM mode for ESI negative and ESI positive compounds for each primary transition, and also for a secondary transition for each analyte for positive confirmation (Table S3).

An isotopically labeled version of each analyte, corresponding to the isotopes added to each sample prior to extraction, was added to each calibration point to generate a relative response ratio. Recoveries of the isotopes were compared with the relative response ratio and a concentration of the unlabeled analyte was calculated. Isotope peak areas for each analyte in every sample were compared to the average isotope peak areas in calibrations, with data reporting limited to sample peak areas above 10% when compared to calibrations. Linear or quadratic regression with 1/x weighting was used and regression coefficients typically exceeded 0.995. Calibration curve verifications were analyzed at least every six samples and were generally between 80% and 120% of the expected concentration. Sample extracts with compound concentrations greater than the calibration range were diluted in 70% methanol and reanalyzed. All reported aqueous values accounted for sample-specific dilution or concentration.

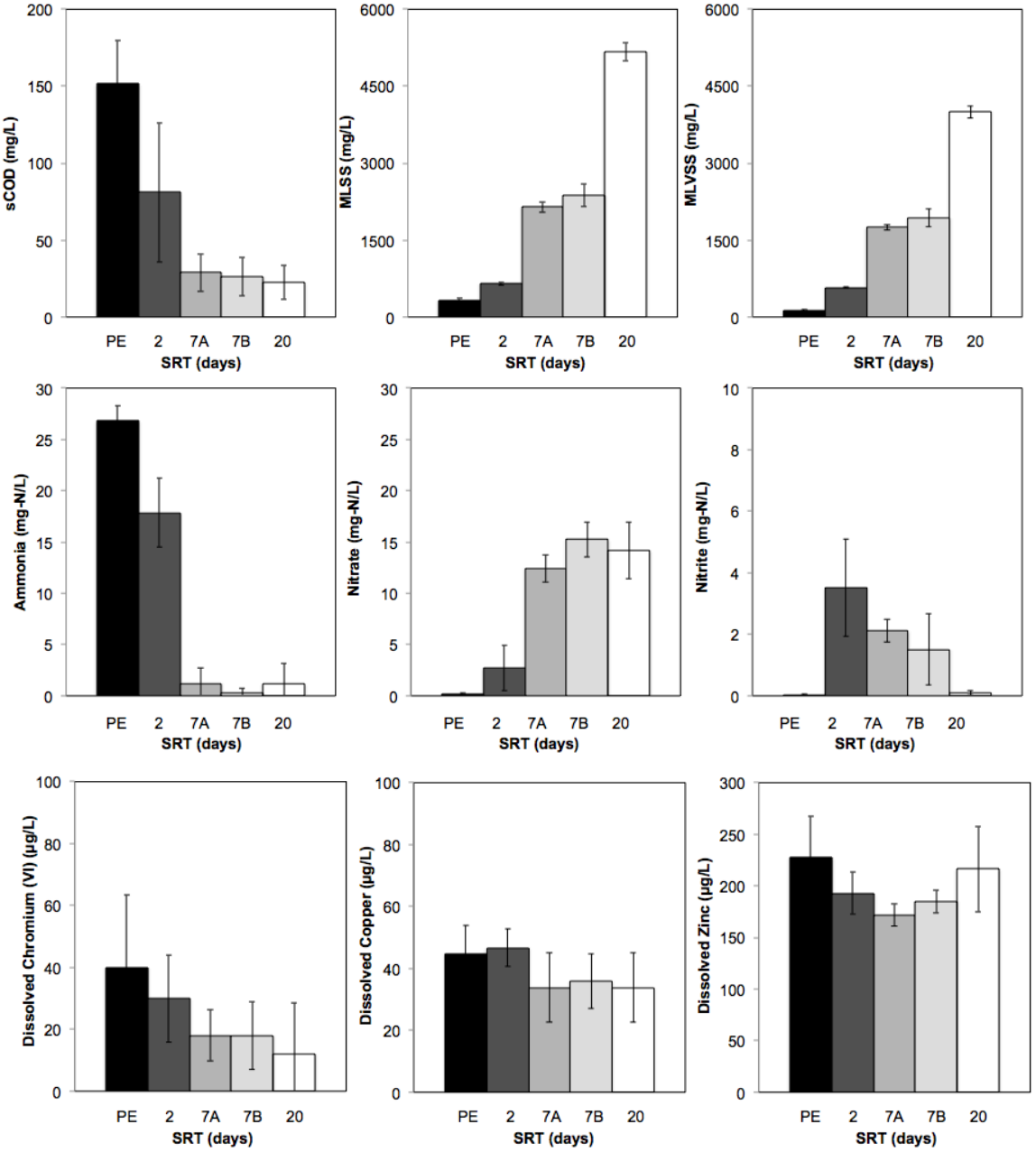
Table S4. Antibiotic concentrations (μg/mL) used for the MIC assays and additional details related to selection and preparation of the media used for the microbiology assays.

MIC	AMP	SMX/TMP	TET	VA
0x	0	0	0	0
0.5x	16	38/2	8	2
1x	32	76/4	16	4
2x	64	152/8	32	8
4x	128	304/16	64	16
8x	256	608/32	128	32
16x	512	1216/64	256	64
32x	1024	2432/128	512	128

Supporting text for selection of Mueller Hinton agar: Mueller Hinton broth and agar are often used for antibiotic susceptibility testing because it has a high buffering capacity that reduces the potential for chemical transformation, thereby ensuring maximum efficacy of the added antibiotics. MH media also contains reduced concentrations of para-aminobenzoic acid (pABA) and thymine/thymidine. These compounds have been shown to reduce the efficacy of sulfonamides and trimethoprim, respectively (Amyes et al., 1974; Neyestani et al., 2017).

Supporting text for preparation of antibiotic stock solutions: Solvents included 0.1 M PBS at pH 8.0 for ampicillin; 50% (v/v) sterile nanopure hot water and a minimal amount of 2.5 M NaOH for sulfamethoxazole; 90% (v/v) sterile nanopure water and 10% (v/v) 0.05 M hydrochloric acid for trimethoprim; and sterile nanopure water for tetracycline and vancomycin. The antibiotic stock solutions were then passed through 0.1-μm acrodisc syringe filters (PALL, Port Washington, NY) for filter sterilization. Diluents included 0.1 M PBS at pH 6.0 for ampicillin and water for sulfamethoxazole, trimethoprim, tetracycline and vancomycin.

Figure S3. Summary of general water quality parameters, including sCOD, MLSS, MLVSS, nitrogen speciation, and metals for the phase 1 testing of solids retention time. Each column represents the mean of 3-5 sample sets with error bars representing ±1 standard deviation.



Supporting text: Some general water quality results were described previously (Neyestani et al., 2017) but are also summarized here. Suspended solids concentrations were consistent with the target SRTs, with MLSS averaging 654±33 mg/L, 2142±104 mg/L, 2374±219 mg/L, and 5172±182 mg/L for SRTs of 2 days, 7(A) days, 7(B) days, and 20 days, respectively. MLVSS values were approximately 80% of the MLSS values. The average pH of the primary effluent was 6.4±0.2, and the pH of the SBR effluent was relatively constant, regardless of SRT, with an average of 6.9±0.2. During the aeration phase, the average dissolved oxygen concentration was

relatively constant in the four SBRs with an average of 4.7±0.5 mg/L and no reading lower than 3.7 mg/L. Average sCOD values over the 60-day test period were 152±28 mg/L for the primary effluent, 81±45 mg/L for the 2-day SRT, 29±12 mg/L for the 7(A)-day SRT, 26±13 mg/L for the 7(B)-day SRT, and 22±11 mg/L for the 20-day SRT.

The ambient temperature during phase 1 dropped from 30°C to 10°C, which had only a minor impact on sCOD removal. However, nitrification was severely inhibited once the temperature dropped to 10°C, although limited ammonia conversion was still observed for the SBRs operating with SRTs of 7 and 20 days. The effects of the temperature change on general water quality parameters are illustrated in greater detail in Neyestani et al. (2017), and the effects of the temperature change on antibiotic resistance are described in greater detail in the main text.

Figure S3 also illustrates the average concentrations of dissolved chromium (VI) (24±18 μg/L), copper (39±10 μg/L), and zinc (199±33 μg/L) in the primary effluent and SBR effluents over the 60-day SRT testing phase. Of the three metals, zinc was consistently present at the highest concentration, which might be a byproduct of the 1 mg/L of zinc orthophosphate that is added to the local drinking water for corrosion control in the distribution system. Some trace metals, including zinc, nickel, chromium, copper, cobalt, vanadium, and tungsten, are important for bacterial metabolic activity and enzyme production, but these metals can also be toxic at elevated concentrations (Seiler and Berendonk, 2012). Some studies indicate that concentrations of copper and zinc as low as 0.05 and 20 μg/L, respectively, are capable of promoting co-selection of bacterial antibiotic resistance (Seiler and Berendonk, 2012; Stepanauskas et al., 2005), while another study noted that concentrations >60 mg/L promote resistance to sulfamethoxazole, ciprofloxacin, and other antibiotics (Becerra-Castro et al., 2015). Therefore, it is not entirely clear whether metals must be present at the μg/L level or the mg/L level to contribute to the development of antibiotic resistance. Additional MIC-type testing would be necessary to fully characterize the role of metals in promoting antibiotic resistance in wastewater treatment applications, but this was not the focus of the current study.

Table S5. Summary of plate counts for the primary effluent and mixed liquor suspended solids as a function of solids retention time (SRT) (i.e., phase 1). (Top) Sample event 1: 3-5 days post-startup; (Middle) sample event 2: 31-33 days post-startup; (Bottom) sample event 3: 53-55 days post-startup. The data represent averages of triplicate plates ±1 standard deviation. The 'total' culturable Staph/Strep were quantified on Mueller Hinton (MH) agar with Staph/Strep (S/S) selective supplement. The antibiotic resistant Staph/Strep were quantified on the same agar but also supplemented with the specified antibiotic(s) at the baseline minimum inhibitory concentrations. The data are separated into two groups because the antibiotics were tested on two different days with different samples, as designated by the two sets of "MH+S/S" data.

Sample	MH+S/S (CFU/100 μL)	MH+S/S+ AMP (CFU/100 μL)	MH+S/S+ SMX/TMP (CFU/100 μL)	MH+S/S (CFU/100 μL)	MH+S/S+ TET (CFU/100 μL)	MH+S/S+ VA (CFU/100 μL)
Primary Effluent	$(8.70\pm0.46)\times10^{3}$	$(7.23\pm0.15)\times10^{1}$	$(6.13\pm0.59)\times10^{1}$	$(4.87\pm0.40)\times10^{3}$	$(6.33\pm0.49)\times10^{1}$	$(3.47\pm0.15)\times10^{1}$
SRT = 2 Days	$(5.60\pm0.72)\times10^{3}$	$(1.02\pm0.06)\times10^{3}$	$(1.05\pm0.05)\times10^{3}$	$(1.90\pm0.06)\times10^{4}$	$(1.38\pm0.08)\times10^{3}$	$(1.90\pm0.10)\times10^{3}$
SRT = 7 Days (A)	$(7.13\pm0.86)\times10^{3}$	$(1.37\pm0.08)\times10^{3}$	$(1.86\pm0.05)\times10^{3}$	$(2.63\pm0.40)\times10^{4}$	$(2.17\pm0.12)\times10^{3}$	$(3.53\pm0.31)\times10^{3}$
SRT = 7 Days (B)	$(5.10\pm0.17)\times10^{3}$	$(1.38\pm0.03)\times10^{3}$	$(1.13\pm0.07)\times10^{3}$	$(2.77\pm0.06)\times10^{4}$	$(2.40\pm0.17)\times10^{3}$	$(3.43\pm0.32)\times10^{3}$
SRT = 20 Days	$(3.13\pm0.29)\times10^{4}$	$(1.05\pm0.05)\times10^{4}$	$(9.47\pm0.81)\times10^{3}$	$(8.37\pm0.35)\times10^{4}$	$(8.20\pm0.17)\times10^{3}$	$(1.20\pm0.03)\times10^{4}$

Sample	MH+S/S (CFU/100 μL)	MH+S/S+ AMP (CFU/100 μL)	MH+S/S+ SMX/TMP (CFU/100 μL)	MH+S/S (CFU/100 μL)	MH+S/S+ TET (CFU/100 μL)	MH+S/S+ VA (CFU/100 μL)
Primary Effluent	$(1.67\pm0.40)\times10^{4}$	$(2.33\pm0.32)\times10^{2}$	$(2.97\pm0.57)\times10^{2}$	$(2.30\pm0.44)\times10^{4}$	$(2.07\pm0.29)\times10^{2}$	$(5.00\pm0.62)\times10^{2}$
SRT = 2 Days	$(1.90\pm0.17)\times10^{4}$	$(4.33\pm0.76)\times10^{2}$	$(4.87\pm0.51)\times10^{2}$	$(2.57\pm0.71)\times10^{4}$	$(2.73\pm0.50)\times10^{2}$	$(3.80\pm0.60)\times10^{3}$
SRT = 7 Days (A)	$(5.57\pm0.61)\times10^{4}$	$(2.67\pm0.25)\times10^{3}$	$(7.10\pm0.66)\times10^{3}$	$(2.90\pm0.46)\times10^{4}$	$(4.13\pm0.29)\times10^{2}$	$(5.57\pm0.64)\times10^{3}$
SRT = 7 Days (B)	$(5.30\pm0.27)\times10^{4}$	$(1.93\pm0.25)\times10^{3}$	$(5.80\pm0.40)\times10^{3}$	$(3.57\pm0.40)\times10^{4}$	$(3.80\pm0.53)\times10^{2}$	$(6.33\pm0.68)\times10^{3}$
SRT = 20 Days	$(8.50\pm0.69)\times10^{4}$	$(8.93\pm0.15)\times10^{3}$	$(1.24\pm0.06)\times10^{4}$	$(3.93\pm0.32)\times10^{4}$	$(1.83\pm0.25)\times10^{3}$	$(1.01\pm0.08)\times10^{4}$

Sample	MH+S/S (CFU/100 μL)	MH+S/S+ AMP (CFU/100 μL)	MH+S/S+ SMX/TMP (CFU/100 μL)	MH+S/S (CFU/100 μL)	MH+S/S+ TET (CFU/100 μL)	MH+S/S+ VA (CFU/100 μL)
Primary Effluent	$(3.67\pm0.85)\times10^{4}$	$(1.37\pm0.21)\times10^{2}$	$(4.23\pm0.40)\times10^{2}$	$(3.90\pm0.60)\times10^{4}$	$(3.90\pm0.60)\times10^{1}$	$(6.23\pm0.50)\times10^{2}$
SRT = 2 Days	$(5.57\pm0.32)\times10^{4}$	$(4.87\pm1.00)\times10^{2}$	$(1.17\pm0.15)\times10^{3}$	$(5.97\pm0.47)\times10^{4}$	$(2.07\pm0.25)\times10^{2}$	$(8.67\pm1.15)\times10^{3}$
SRT = 7 Days (A)	$(6.70\pm0.36)\times10^{4}$	$(9.37\pm0.60)\times10^{2}$	$(4.57\pm2.20)\times10^{3}$	$(8.43\pm0.59)\times10^{4}$	$(3.73\pm0.50)\times10^{2}$	$(1.40\pm0.27)\times10^{4}$
SRT = 7 Days (B)	$(6.57\pm0.45)\times10^{4}$	$(1.02\pm0.07)\times10^{3}$	$(4.43\pm0.76)\times10^{3}$	$(8.60\pm0.46)\times10^{4}$	$(4.23\pm0.45)\times10^{2}$	$(1.43\pm0.35)\times10^{4}$
SRT = 20 Days	$(1.25\pm0.06)\times10^{5}$	$(4.23\pm0.31)\times10^{3}$	$(1.03\pm0.06)\times10^{4}$	$(9.97\pm0.25)\times10^{4}$	$(2.43\pm0.67)\times10^{3}$	$(2.30\pm0.27)\times10^{4}$

Supporting text: Colonies that grew in the absence of antibiotics were considered the 'total' culturable Gram positive cocci in each sample, while colonies that grew in the presence of the antibiotics at the baseline MICs were described as being 'antibiotic resistant' for this research. The AR bacteria were reported as a ratio to the 'total' counts to provide a measure of relative prevalence, thereby adjusting for differences in population size between experimental conditions. Three sample events were performed for each phase of the SBR research, and each sample event was divided into two different sample groups to limit the plating on each day. Resistance to AMP and SMX/TMP was generally tested on the first day of each sample event, and TET and VA were generally tested on the second day of each sample event, although the order was switched for the third sample event to reduce potential bias. The 'total' culturable count was determined on both days.

Table S6. Summary of changes in Staph/Strep abundance and antibiotic resistance percentages from sample event 1 (3-5 days post-startup) of the SRT testing phase. The data are separated into two groups because the antibiotics were tested on two different days with different samples.

Sample	Group 1 Ratio of MLSS/PE (no antibiotics)	AMP Resistant (%)	SMX/TMP Resistant (%)	Group 2 Ratio of MLSS/PE (no antibiotics)	TET Resistant (%)	VA Resistant (%)
Primary Effluent	--	1%	1%	--	1%	1%
SRT = 2 Days	0.64	18%	19%	3.91	7%	10%
SRT = 7 Days (A)	0.82	19%	26%	5.41	8%	13%
SRT = 7 Days (B)	0.59	27%	22%	5.68	9%	12%
SRT = 20 Days	3.60	34%	30%	17.19	10%	14%

Figure S4. Summary of antibiotic resistance percentages from sample event 1 (3-5 days post-startup) of the SRT testing phase. Columns represent the means of triplicate plates, and error bars represent ±1 standard deviation.

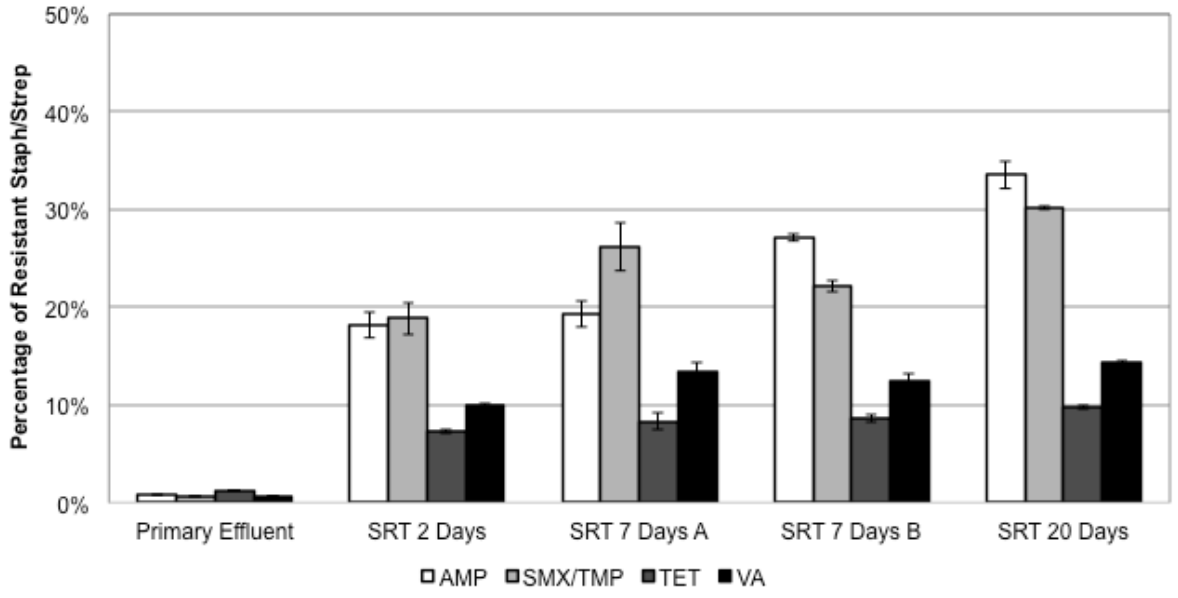


Table S7. Summary of changes in Staph/Strep abundance and antibiotic resistance percentages from sample event 2 (31-33 days post-startup) of the SRT testing phase. The data are separated into two groups because the antibiotics were tested on two different days with different samples.

Sample	Group 1 Ratio of MLSS/PE (no antibiotics)	AMP Resistant (%)	SMX/TMP Resistant (%)	Group 2 Ratio of MLSS/PE (no antibiotics)	TET Resistant (%)	VA Resistant (%)
Primary Effluent	--	1%	2%	--	1%	2%
SRT = 2 Days	1.14	2%	3%	1.12	1%	15%
SRT = 7 Days (A)	3.34	5%	13%	1.26	1%	19%
SRT = 7 Days (B)	3.18	4%	11%	1.55	1%	18%
SRT = 20 Days	5.10	11%	15%	1.71	5%	26%

Figure S5. Summary of antibiotic resistance percentages from sample event 2 (31-33 days post-startup) of the SRT testing phase. Columns represent the means of triplicate plates, and error bars represent ±1 standard deviation.

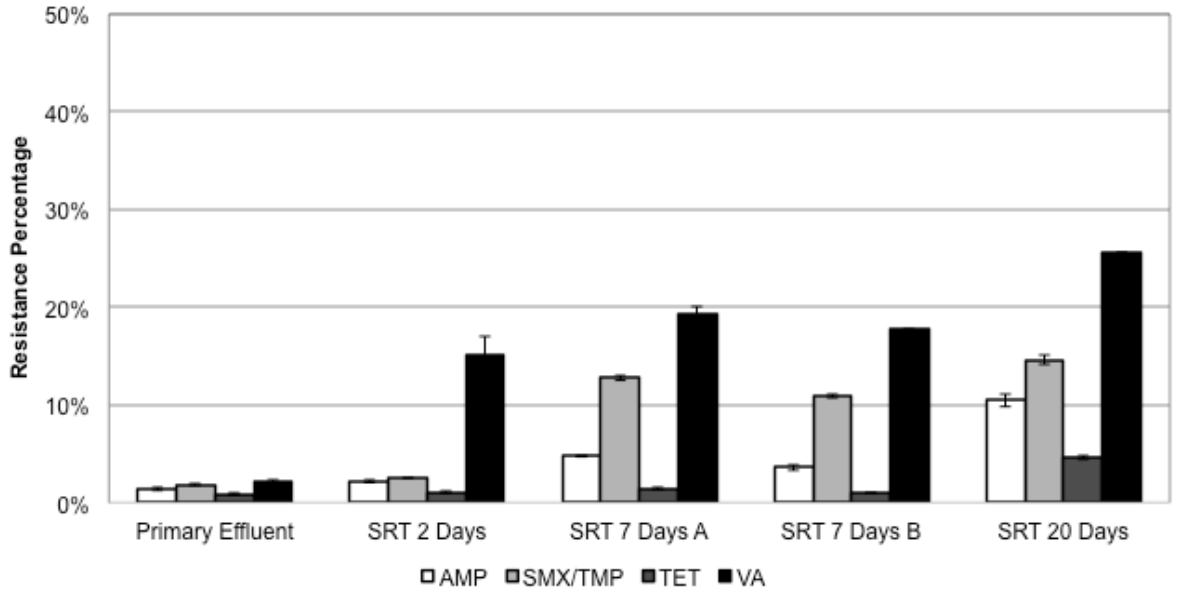


Table S8. Summary of changes in Staph/Strep abundance and antibiotic resistance percentages from sample event 3 (53-55 days post-startup) of the SRT testing phase. The data are separated into two groups because the antibiotics were tested on two different days with different samples.

Sample	Group 1 Ratio of MLSS/PE (no antibiotics)	AMP Resistant (%)	SMX/TMP Resistant (%)	Group 2 Ratio of MLSS/PE (no antibiotics)	TET Resistant (%)	VA Resistant (%)
Primary Effluent	--	0%	1%	--	0%	2%
SRT = 2 Days	1.52	1%	2%	1.53	0%	15%
SRT = 7 Days (A)	1.83	1%	7%	2.16	0%	17%
SRT = 7 Days (B)	1.79	2%	7%	2.21	0%	17%
SRT = 20 Days	3.42	3%	8%	2.56	2%	23%

Figure S6. Summary of antibiotic resistance percentages from sample event 3 (53-55 days post-startup) of the SRT testing phase. Columns represent the means of triplicate plates, and error bars represent ±1 standard deviation.

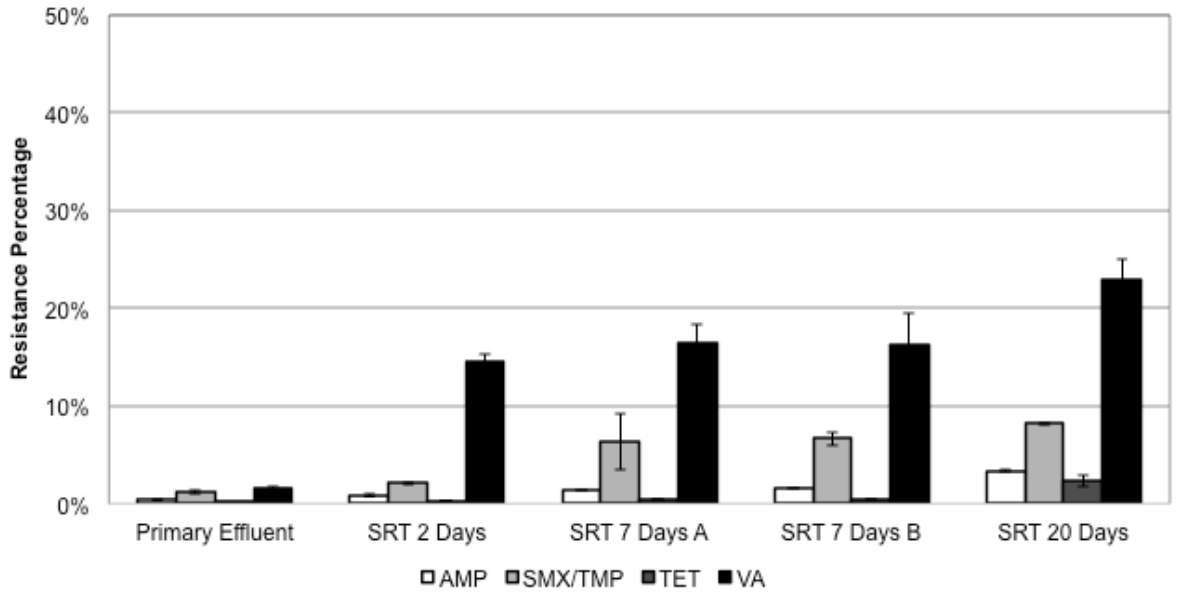


Table S9. Summary of ANOVA results from the SRT experiments.

Antibiotic	Sample Event 1		Sample Event 2		Sample Event 3	
	Not Different	Different	Not Different	Different	Not Different	Different
AMP	None	PE and 2 PE and 7A PE and 7B PE and 20 2 and 7A 2 and 7B 2 and 20 7A and 7B 7A and 20 7B and 20	PE and 2	PE and 7A PE and 7B PE and 20 2 and 7A 2 and 7B 2 and 20 7A and 7B 7A and 20 7B and 20	7A and 7B	PE and 2 PE and 7A PE and 7B PE and 20 2 and 7A 2 and 7B 2 and 20 7A and 7B 7A and 20 7B and 20
SMX/TMP	2 and 7B	PE and 2 PE and 7A PE and 7B PE and 20 2 and 7A 2 and 20 7A and 7B 7A and 20 7B and 20	PE and 2 7A and 7B 7A and 20	PE and 7A PE and 7B PE and 20 2 and 7A 2 and 7B 2 and 20 7B and 20	PE and 2 7A and 7B 7A and 20 7B and 20	PE and 7A PE and 7B PE and 20 2 and 7A 2 and 7B 2 and 20
TC	7A and 20	PE and 2 PE and 7A PE and 7B PE and 20 2 and 7A 2 and 7B 2 and 20 7A and 7B 7B and 20	PE and 2 PE and 7A PE and 7B 2 and 7A 2 and 7B 7A and 7B	PE and 20 2 and 20 7A and 20 7B and 20	PE and 2 PE and 7A PE and 7B 2 and 7A 2 and 7B 7A and 7B	PE and 20 2 and 20 7A and 20 7B and 20
VA	7A and 20	PE and 2 PE and 7A PE and 7B PE and 20 2 and 7A 2 and 7B 2 and 20 7A and 7B 7B and 20	2 and 7A 2 and 7B 7A and 7B	PE and 2 PE and 7A PE and 7B PE and 20 2 and 7A 2 and 7B 2 and 20 7A and 7B 7A and 20 7B and 20	2 and 7A 2 and 7B 7A and 7B 7A and 20 7B and 20	PE and 2 PE and 7A PE and 7B PE and 20 2 and 7A 2 and 7B 2 and 20 7A and 7B

[1]Statistical significance determined at $\alpha = 0.05$

Table S10. Summary of MIC data for the primary effluent during the SRT testing phase.

SRT	Antibiotic	Sample Event	% Revived	% AR	Baseline MIC	Observed MIC and Number of Isolates								Weighted Score	Avg. Score	St. Dev.	Avg. Score	St. Dev.
					(µg/mL)	<1x	1x	2x	4x	8x	16x	32x	>32x					
Primary Effluent	AMP	1	100%	100%	32	0	0	0	0	0	0	0	8	64.0	58.8	8.9	54.3	19.0
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	50%	100%		0	0	1	0	0	0	0	3	48.5				
	SMX/TMP	1	75%	100%	76/4	0	0	2	1	0	0	0	3	33.3	33.1	31.0		
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	13%	100%		0	0	1	0	0	0	0	0	2.0				
	TET	1	100%	100%	16	0	0	1	0	0	0	0	7	56.3	61.4	4.5		
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	88%	100%		0	0	0	0	0	0	0	7	64.0				
	VA	1	100%	100%	4	0	0	0	0	0	0	0	8	64.0	64.0	0.0		
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	100%	100%		0	0	0	0	0	0	0	8	64.0				

Table S11. Summary of MIC data for the SBR with a 2-day SRT during the SRT testing phase.

SRT (d)	Antibiotic	Sample Event	% Revived	% AR	Baseline MIC	Observed MIC and Number of Isolates								Weighted Score	Avg. Score	St. Dev.	Avg. Score	St. Dev.
					(µg/mL)	<1x	1x	2x	4x	8x	16x	32x	>32x					
2	AMP	1	88%	100%	32	0	0	0	0	0	0	0	7	64.0	43.3	35.8	54.8	20.9
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	13%	100%		0	0	1	0	0	0	0	0	2.0				
	SMX/TMP	1	100%	100%	76/4	0	0	0	0	0	0	0	8	64.0	64.0	0.0		
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	88%	100%		0	0	0	0	0	0	0	7	64.0				
	TET	1	100%	100%	16	0	0	0	0	0	0	0	8	64.0	64.0	0.0		
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	100%	100%		0	0	0	0	0	0	0	8	64.0				
	VA	1	100%	100%	4	0	0	0	0	0	0	0	8	64.0	48.0	24.3		
		2	100%	100%		0	0	2	1	3	0	0	2	20.0				
		3	100%	100%		0	0	0	0	0	0	1	7	60.0				

Table S12. Summary of MIC data for the SBR with a 7-day (A) SRT during the SRT testing phase.

SRT (d)	Antibiotic	Sample Event	% Revived	% AR	Baseline MIC	Observed MIC and Number of Isolates								Weighted Score	Avg. Score	St. Dev.	Avg. Score	St. Dev.
					(µg/mL)	<1x	1x	2x	4x	8x	16x	32x	>32x					
7A	AMP	1	100%	100%	32	0	0	1	0	0	0	0	7	56.3	61.4	4.5	56.1	13.5
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	25%	100%		0	0	0	0	0	0	0	2	64.0				
	SMX/TMP	1	100%	100%	76/4	0	0	0	0	0	0	0	8	64.0	57.1	11.9		
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	38%	100%		0	0	1	0	0	0	0	2	43.3				
	TET	1	88%	100%	16	0	0	1	0	0	0	0	6	55.1	59.0	4.5		
		2	100%	100%		0	0	0	0	0	1	0	7	58.0				
		3	100%	100%		0	0	0	0	0	0	0	8	64.0				
	VA	1	100%	100%	4	0	0	2	0	0	0	0	6	48.5	41.7	26.4		
		2	100%	100%		0	0	2	4	0	1	0	1	12.5				
		3	75%	100%		0	0	0	0	0	0	0	6	64.0				

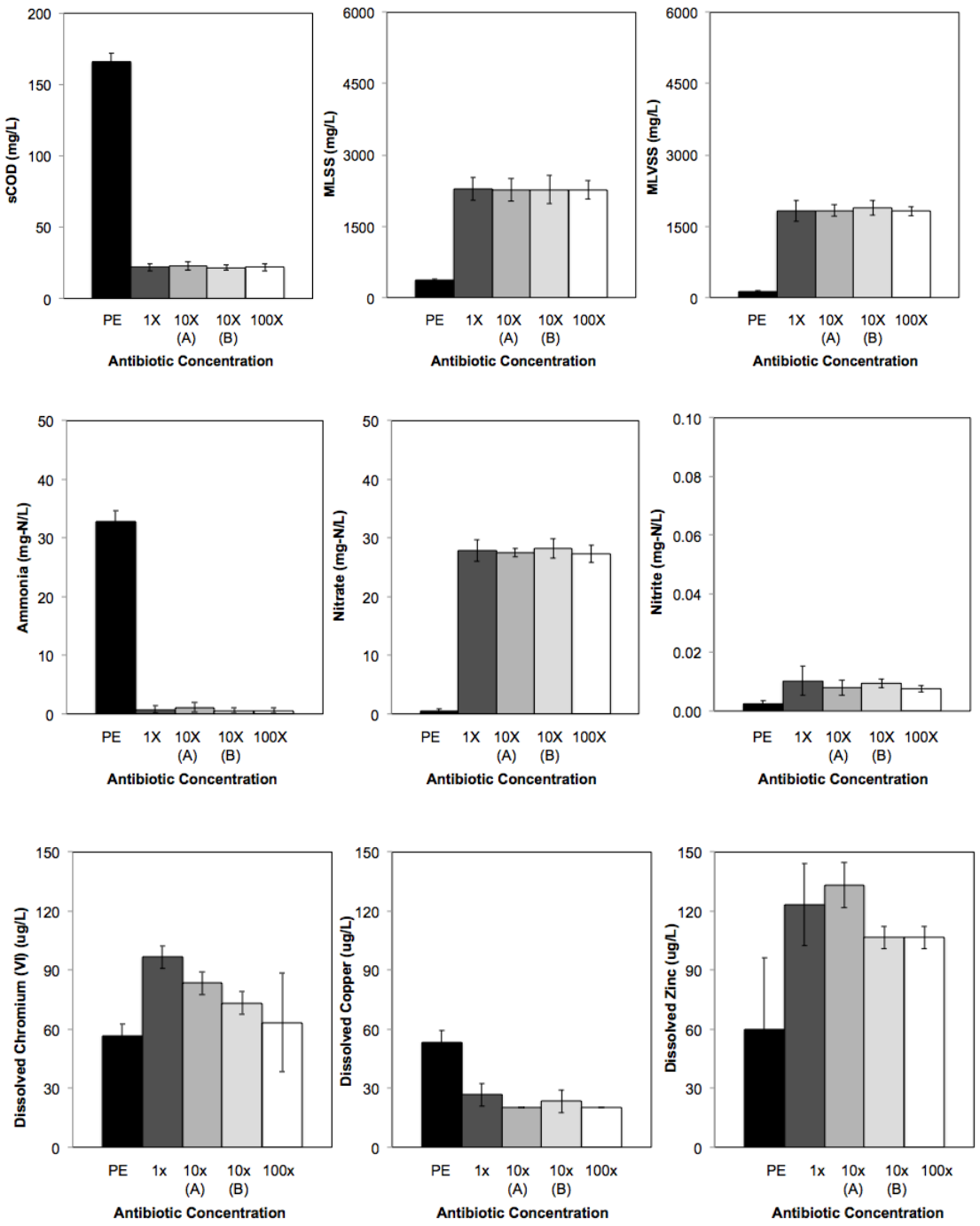
Table S13. Summary of MIC data for the SBR with a 7-day (B) SRT during the SRT testing phase.

SRT (d)	Antibiotic	Sample Event	% Revived	% AR	Baseline MIC	Observed MIC and Number of Isolates								Weighted Score	Avg. Score	St. Dev.	Avg. Score	St. Dev.
					(µg/mL)	<1x	1x	2x	4x	8x	16x	32x	>32x					
7B	AMP	1	50%	100%	32	0	0	0	0	0	0	0	4	64.0	58.8	8.9	56.8	11.5
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	50%	100%		0	0	1	0	0	0	0	3	48.5				
	SMX/TMP	1	88%	100%	76/4	0	0	0	0	0	0	0	7	64.0	57.1	11.9		
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	75%	100%		0	0	2	0	0	0	0	4	43.3				
	TET	1	100%	100%	16	0	0	2	1	0	0	0	5	41.0	56.3	13.3		
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	100%	100%		0	0	0	0	0	0	0	8	64.0				
	VA	1	100%	100%	4	0	0	3	0	0	0	0	5	40.8	45.3	16.8		
		2	100%	100%		0	0	1	0	1	3	0	3	31.3				
		3	100%	100%		0	0	0	0	0	0	0	8	64.0				

Table S14. Summary of MIC data for the SBR with a 20-day SRT during the SRT testing phase.

SRT (d)	Antibiotic	Sample Event	% Revi-ved	% AR	Baseline MIC	Observed MIC and Number of Isolates								Weight-ed Score	Avg. Score	St. Dev.	Avg. Score	St. Dev.
					(µg/mL)	<1x	1x	2x	4x	8x	16x	32x	>32x					
20	AMP	1	38%	100%	32	0	0	0	0	0	0	0	3	64.0	64.0	0.0	61.8	4.6
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	38%	100%		0	0	0	0	0	0	0	3	64.0				
	SMX/TMP	1	75%	100%	76/4	0	0	0	0	0	0	0	6	64.0	64.0	0.0		
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	50%	100%		0	0	0	0	0	0	0	4	64.0				
	TET	1	100%	100%	16	0	0	0	0	0	0	1	7	60.0	62.7	2.3		
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	100%	100%		0	0	0	0	0	0	0	8	64.0				
	VA	1	100%	100%	4	0	0	1	0	1	0	0	6	49.3	56.6	7.4		
		2	100%	100%		0	0	0	1	0	0	0	7	56.5				
		3	88%	100%		0	0	0	0	0	0	0	7	64.0				

Figure S7. Summary of general water quality parameters, including sCOD, MLSS, MLVSS, nitrogen speciation, and metals for the phase 2 testing of influent antibiotic concentrations. Each column represents the mean of 3 sample sets with error bars representing ±1 standard deviation.



Supporting text: Over the duration of phase 2, the pH of the primary effluent was consistently 6.8, and the pH of the four reactors was consistently 7.1-7.2. The average DO concentrations were 3.7-3.8 in the four reactors, with no reading lower than 3.4 mg/L. Figure S7 provides a

summary of the other general water quality parameters, including sCOD, total and volatile suspended solids concentrations in the primary effluent and mixed liquors (i.e., MLSS and MLVSS), nitrogen speciation, and metals. Because phase 2 maintained an SRT of ~7 days in all four reactors, the MLSS and MLVSS values were nearly identical in the four reactors, with an overall average MLSS of 2,274±43 mg/L and an overall average MLVSS of 1,840±53 mg/L. Stable operation of the SBRs resulted in an overall average sCOD reduction of 87% and an overall average sCOD of 22±0.40 mg/L in the SBR effluents. There were no apparent differences between the reactors with respect to nitrification. All reactors were operated with an SRT of ~7 days, which is sufficient to maintain a stable population of nitrifiers. As a result, the ammonia in the primary effluent (average of 32.8±1.8 mg-N/L) was consistently converted to nitrate (overall average of 27.7±0.5 mg-N/L) and, to a much lesser extent, nitrite (overall 0.01±0.00 mg-N/L). The residual ammonia concentration in the four reactors was an average of 0.7±0.2 mg-N/L. There were no apparent differences in the concentrations of chromium (VI) (79±17 μg/L), copper (23±4.5 μg/L), or zinc (118±16 μg/L) in the SBR effluents so it is unlikely that these metals caused any differences in antibiotic resistance between the reactors. These data indicate that the elevated, albeit sub-inhibitory, antibiotic concentrations did not impact overall treatment performance with respect to general water quality parameters.

Table S15. Summary of TOrC concentrations during phase 2 (varying influent antibiotic concentrations).

TOrC	Unit	Sample Event 3A (5/12/2016)									Sample Event 3B (5/13/2016)								
		PE1	1x	1x[1]	PE2	10xA	PE3	10xB	PE4	100x	PE1	1x	PE2	10xA	PE3	10xB	PE4	100x	100x[1]
Ampicillin	ng/L	<100	<100	<100	<100	<100	<10000	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100
Sulfamethoxazole	ng/L	2700	2000	2000	12000	6300	11000	6100	92000	51000	2300	1500	11000	5600	11000	5900	100000	59000	60000
Tetracycline	ng/L	<100	23	<100	<100	26	<10000	25	390	160	<100	16	<100	24	<100	30	140	160	160
Trimethoprim	ng/L	980	110	89	4800	1300	5000	1300	45000	20000	910	220	4800	1500	4300	850	48000	23000	23000
Vancomycin	ng/L	1200	1100	1100	2100	2000	6500	2100	33000	23000	920	1300	1700	2300	1800	2200	28000	26000	28000
Acetaminophen	ng/L	100000	<100	<100	96000	<100	100000	<100	98000	<100	90000	<100	91000	<100	90000	<100	94000	<100	<100
Atenolol	ng/L	1200	120	120	1200	130	1200	160	1200	130	1100	<2000	1100	<2000	1100	<2000	1100	<2000	<2000
Caffeine	ng/L	67000	<5.0	<5.0	66000	<100	64000	<100	63000	<100	70000	<100	66000	<100	64000	<100	65000	<100	<100
Carbamazepine	ng/L	180	180	180	160	170	170	170	170	130	160	150	150	160	150	160	150	160	130
DEET	ng/L	1000	630	620	1100	600	1000	660	1000	500	510	470	490	480	480	390	470	400	340
Fluoxetine	ng/L	<1000	19	18	<1000	16	<1000	16	<1000	16	<1000	13	<1000	22	<1000	21	<1000	22	15
Gemfibrozil	ng/L	1600	7.6	7.5	1600	31	1700	61	1700	41	1500	420	1600	18	1600	16	1600	33	33
Ibuprofen	ng/L	24000	14	17	22000	14	23000	23	23000	27	22000	23	22000	14	22000	13	22000	11	11
Meprobamate	ng/L	780	930	920	800	980	820	950	870	760	710	870	720	910	720	860	750	870	760
Naproxen	ng/L	21000	15	13	20000	14	21000	23	21000	38	19000	36	19000	10	19000	14	19000	19	18
Primidone	ng/L	210	210	200	220	210	240	210	220	170	190	190	190	200	180	190	190	190	180
Sucralose	ng/L	38000	44000	45000	40000	43000	40000	46000	41000	37000	42000	51000	40000	45000	37000	46000	43000	47000	39000
TCEP	ng/L	330	310	300	320	300	290	290	280	280	160	250	160	240	160	230	160	230	240
Triclocarban	ng/L	15	19	12	21	12	20	16	24	28	19	40	16	16	22	18	20	26	28
Triclosan	ng/L	240	36	33	230	46	220	54	210	41	270	150	190	100	240	50	230	42	40

[1]Indicates duplicate sample that was collected and analyzed for quality assurance

Table S16. Summary of plate counts for the primary effluent and mixed liquor suspended solids as a function of influent antibiotic concentrations (i.e., phase 2). (Top) Sample event 1: 3-5 days post-startup; (Middle) sample event 2: 31-33 days post-startup; (Bottom) sample event 3: 14-16 days post-startup (following SBR restart; see text in Figure S1 for more details). The data represent averages of triplicate plate ±1 standard deviation. The 'total' culturable Staph/Strep were quantified on Mueller-Hinton agar with Staph/Strep selective supplement. The antibiotic resistant Staph/Strep were quantified on the same agar but also supplemented with the specified antibiotic(s) at the baseline minimum inhibitory concentrations. The data are separated into two groups because the antibiotics were tested on two different days with different samples, as designated by the two sets of "MH+S/S" data.

Sample	MH+S/S (CFU/100 μL)	MH+S/S+ AMP (CFU/100 μL)	MH+S/S+ SMX/TMP (CFU/100 μL)	MH+S/S (CFU/100 μL)	MH+S/S+ TET (CFU/100 μL)	MH+S/S+ VA (CFU/100 μL)
Primary Effluent	$(2.00\pm0.10)\times10^{4}$	$(1.87\pm0.15)\times10^{2}$	$(5.67\pm0.58)\times10^{2}$	$(1.37\pm0.35)\times10^{4}$	$(1.67\pm0.21)\times10^{2}$	$(9.63\pm0.55)\times10^{2}$
1x	$(1.87\pm0.12)\times10^{4}$	$(5.40\pm0.36)\times10^{2}$	$(7.40\pm0.53)\times10^{2}$	$(2.23\pm0.49)\times10^{4}$	$(4.40\pm0.52)\times10^{2}$	$(2.07\pm0.25)\times10^{3}$
10x (A)	$(1.83\pm0.15)\times10^{4}$	$(2.90\pm0.27)\times10^{3}$	$(1.57\pm0.47)\times10^{3}$	$(2.23\pm0.15)\times10^{4}$	$(7.67\pm0.58)\times10^{2}$	$(3.00\pm0.10)\times10^{3}$
10x (B)	$(1.83\pm0.06)\times10^{4}$	$(2.00\pm0.20)\times10^{3}$	$(1.67\pm0.06)\times10^{3}$	$(2.17\pm0.12)\times10^{4}$	$(7.23\pm0.70)\times10^{2}$	$(3.17\pm0.21)\times10^{3}$
100x	$(1.97\pm0.15)\times10^{4}$	$(2.90\pm0.17)\times10^{3}$	$(1.93\pm0.38)\times10^{3}$	$(1.97\pm0.21)\times10^{4}$	$(8.70\pm0.36)\times10^{2}$	$(3.63\pm0.65)\times10^{3}$

Sample	MH+S/S (CFU/100 μL)	MH+S/S+ AMP (CFU/100 μL)	MH+S/S+ SMX/TMP (CFU/100 μL)	MH+S/S (CFU/100 μL)	MH+S/S+ TET (CFU/100 μL)	MH+S/S+ VA (CFU/100 μL)
Primary Effluent	$(4.07\pm0.50)\times10^{3}$	$(3.17\pm0.51)\times10^{2}$	$(8.00\pm0.36)\times10^{1}$	$(7.67\pm1.53)\times10^{3}$	$(1.40\pm0.36)\times10^{2}$	$(8.07\pm0.21)\times10^{2}$
1x	$(7.00\pm1.00)\times10^{3}$	$(7.33\pm1.53)\times10^{2}$	$(2.80\pm0.40)\times10^{2}$	$(1.33\pm0.25)\times10^{4}$	$(8.67\pm3.21)\times10^{2}$	$(1.83\pm0.32)\times10^{3}$
10x (A)	$(7.67\pm0.58)\times10^{3}$	$(1.13\pm0.12)\times10^{3}$	$(5.70\pm0.44)\times10^{2}$	$(1.37\pm0.25)\times10^{4}$	$(9.67\pm2.08)\times10^{2}$	$(3.23\pm0.21)\times10^{3}$
10x (B)	$(8.33\pm1.53)\times10^{3}$	$(1.27\pm0.31)\times10^{3}$	$(6.43\pm0.57)\times10^{2}$	$(1.27\pm0.31)\times10^{4}$	$(1.07\pm0.35)\times10^{3}$	$(2.93\pm0.15)\times10^{3}$
100x	$(7.67\pm1.53)\times10^{3}$	$(1.73\pm0.21)\times10^{3}$	$(8.60\pm0.70)\times10^{2}$	$(1.30\pm0.35)\times10^{4}$	$(1.47\pm0.35)\times10^{3}$	$(3.73\pm0.12)\times10^{3}$

Sample	MH+S/S (CFU/100 μL)	MH+S/S+ AMP (CFU/100 μL)	MH+S/S+ SMX/TMP (CFU/100 μL)	MH+S/S (CFU/100 μL)	MH+S/S+ TET (CFU/100 μL)	MH+S/S+ VA (CFU/100 μL)
Primary Effluent	$(9.33\pm1.53)\times10^{3}$	$(2.70\pm0.46)\times10^{2}$	$(4.83\pm0.75)\times10^{2}$	$(6.00\pm1.73)\times10^{3}$	$(4.47\pm0.61)\times10^{1}$	$(8.47\pm0.50)\times10^{2}$
1x	$(8.33\pm3.51)\times10^{3}$	$(5.87\pm0.78)\times10^{2}$	$(7.40\pm0.30)\times10^{2}$	$(8.33\pm0.58)\times10^{3}$	$(6.67\pm0.58)\times10^{2}$	$(1.66\pm0.18)\times10^{3}$
10x (A)	$(6.33\pm1.53)\times10^{3}$	$(9.67\pm1.16)\times10^{2}$	$(8.60\pm0.46)\times10^{2}$	$(8.67\pm2.08)\times10^{3}$	$(1.13\pm0.42)\times10^{3}$	$(2.37\pm0.23)\times10^{3}$
10x (B)	$(7.00\pm2.00)\times10^{3}$	$(1.02\pm0.08)\times10^{3}$	$(8.17\pm0.31)\times10^{2}$	$(9.00\pm1.00)\times10^{3}$	$(1.27\pm0.31)\times10^{3}$	$(2.07\pm0.64)\times10^{3}$
100x	$(5.67\pm0.58)\times10^{3}$	$(1.48\pm0.07)\times10^{3}$	$(8.73\pm0.32)\times10^{2}$	$(1.00\pm0.27)\times10^{4}$	$(2.27\pm0.21)\times10^{3}$	$(3.17\pm0.31)\times10^{3}$

Supporting text: Colonies that grew in the absence of antibiotics were considered the 'total' culturable Gram positive cocci in each sample, while colonies that grew in the presence of the antibiotics at the baseline MICs were described as being 'antibiotic resistant' for this research. The AR bacteria were reported as a ratio to the 'total' counts to provide a measure of relative prevalence, thereby adjusting for differences in population size between experimental conditions. Three sample events were performed for each phase of the SBR research, and each sample event was divided into two different sample groups to limit the plating on each day. Resistance to AMP and SMX/TMP was generally tested on the first day of each sample event, and TET and VA were generally tested on the second day of each sample event, although the order was switched for the third sample event to reduce potential bias. The 'total' culturable count was determined on both days.

Table S17. Summary of changes in Staph/Strep abundance and antibiotic resistance percentages from sample event 1 of the antibiotic concentration testing phase. The data are separated into two groups because the antibiotics were tested on two different days with different samples.

Sample	Group 1 Ratio of MLSS/PE (no antibiotics)	AMP Resistant (%)	SMX/TMP Resistant (%)	Group 2 Ratio of MLSS/PE (no antibiotics)	TET Resistant (%)	VA Resistant (%)
Primary Effluent	--	1%	3%	--	1%	7%
1X	0.93	3%	4%	1.63	2%	9%
10X (A)	0.92	16%	9%	1.63	3%	13%
10X (B)	0.92	11%	9%	1.59	3%	15%
100X	0.98	15%	10%	1.44	4%	18%

Figure S8. Summary of antibiotic resistance percentages from sample event 1 of the antibiotic concentration testing phase. Columns represent the means of triplicate plates, and error bars represent ±1 standard deviation.

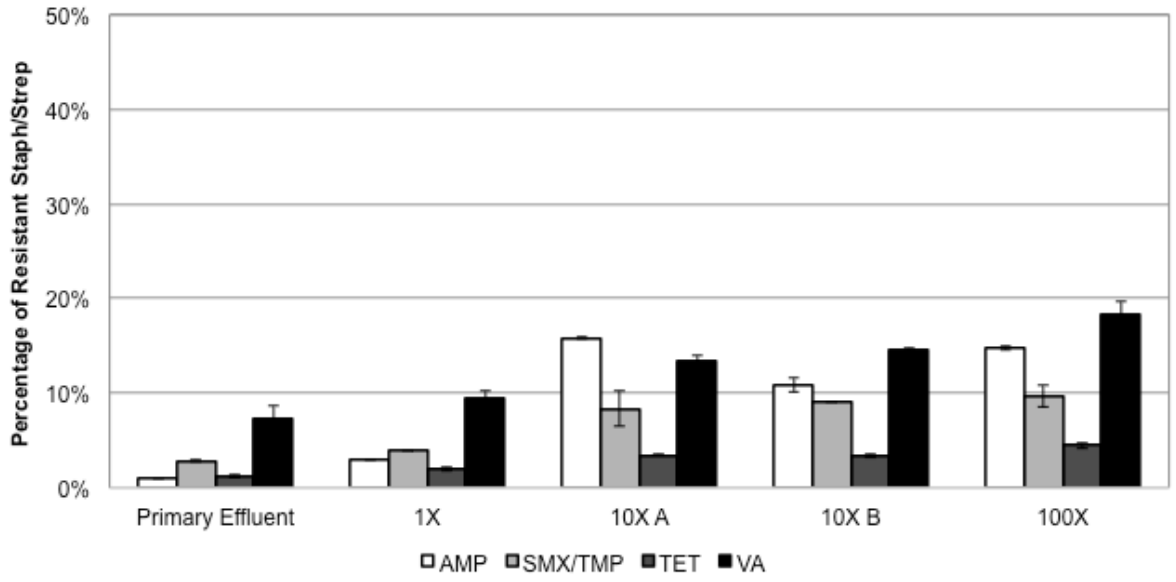


Table S18. Summary of changes in Staph/Strep abundance and antibiotic resistance percentages from sample event 2 of the antibiotic concentration testing phase. The data are separated into two groups because the antibiotics were tested on two different days with different samples.

Sample	Group 1 Ratio of MLSS/PE (no antibiotics)	AMP Resistant (%)	SMX/TMP Resistant (%)	Group 2 Ratio of MLSS/PE (no antibiotics)	TET Resistant (%)	VA Resistant (%)
Primary Effluent	--	8%	2%	--	2%	11%
1X	1.72	10%	4%	1.74	7%	14%
10X (A)	1.89	15%	7%	1.78	7%	24%
10X (B)	2.05	15%	8%	1.65	8%	23%
100X	1.89	23%	11%	1.70	11%	29%

Figure S9. Summary of antibiotic resistance percentages from sample event 2 of the antibiotic concentration testing phase. Columns represent the means of triplicate plates, and error bars represent ±1 standard deviation.

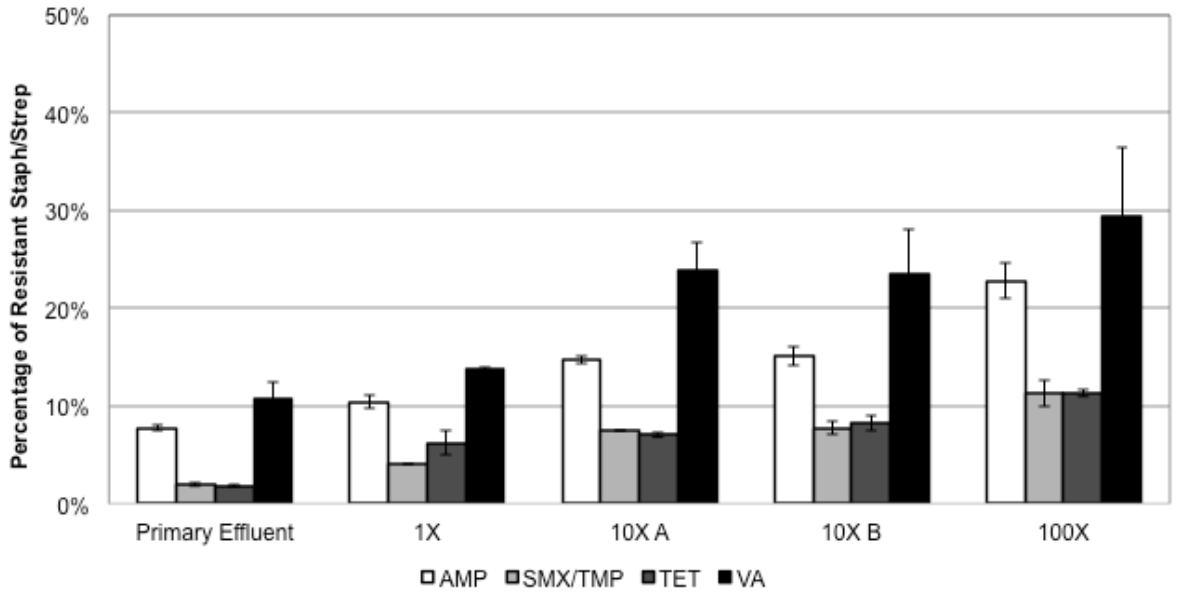


Table S19. Summary of changes in Staph/Strep abundance and antibiotic resistance percentages from sample event 3 of the antibiotic concentration testing phase. The data are separated into two groups because the antibiotics were tested on two different days with different samples.

Sample	Group 1 Ratio of MLSS/PE (no antibiotics)	AMP Resistant (%)	SMX/TMP Resistant (%)	Group 2 Ratio of MLSS/PE (no antibiotics)	TET Resistant (%)	VA Resistant (%)
Primary Effluent	--	3%	5%	--	1%	14%
1X	0.89	7%	9%	1.39	8%	20%
10X (A)	0.68	15%	14%	1.44	13%	27%
10X (B)	0.75	15%	12%	1.50	14%	23%
100X	0.61	26%	15%	1.67	23%	32%

Figure S10. Summary of antibiotic resistance percentages from sample event 3 of the antibiotic concentration testing phase. Columns represent the means of triplicate plates, and error bars represent ±1 standard deviation.

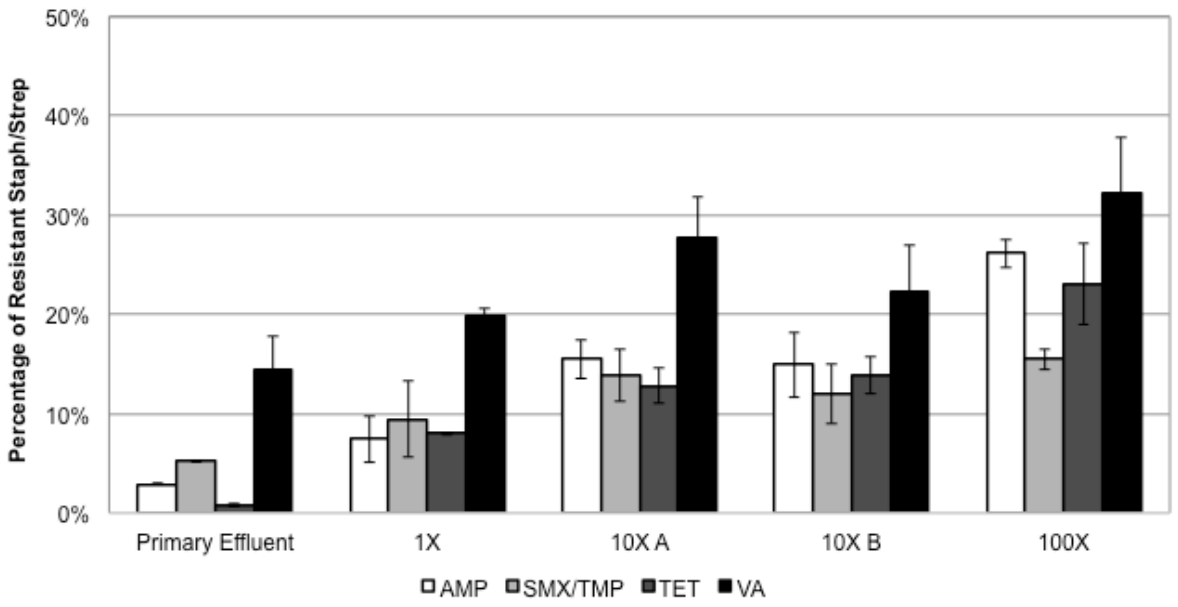


Table S20. Summary of ANOVA results from the antibiotic concentration experiments.

Antibiotic	Sample Event 1		Sample Event 2		Sample Event 3	
	Not Different	Different	Not Different	Different	Not Different	Different
AMP	PE | 1x 10xA | 100x	PE | 10xA PE | 10xB PE | 100x 1x | 10xA 1x | 10xB 1x | 100x 10xA | 10xB 10xB | 100x	PE | 1x 1x | 10xA 1x | 10xB 10xA | 10xB	PE | 10xA PE | 10xB PE | 100x 1x | 100x 10xA | 100x 10xB | 100x	10xA | 10xB	PE | 1x PE | 10xA PE | 10xB PE | 100x 1x | 10xA 1x | 10xB 1x | 100x 10xA | 100x 10xB | 100x
SMX/TMP	PE | 1x 10xA | 10xB 10xA | 100x 10xB | 100x	PE | 10xA PE | 10xB PE | 100x 1x | 10xA 1x | 10xB 1x | 100x	10xA | 10xB	PE | 1x PE | 10xA PE | 10xB PE | 100x 1x | 10xA 1x | 10xB 1x | 100x 10xA | 100x 10xB | 100x	10xA | 100x	PE | 1x PE | 10xA PE | 10xB PE | 100x 1x | 10xA 1x | 10xB 1x | 100x 10xA | 10xB 10xB | 100x
TC	10xA | 10xB	PE | 1x PE | 10xA PE | 10xB PE | 100x 1x | 10xA 1x | 10xB 1x | 100x 10xA | 100x 10xB | 100x	PE | 1x PE | 10xA 1x | 10xA 1x | 10xB 10xA | 10xB 10xA | 100x 10xB | 100x	PE | 10xB PE | 100x 1x | 100x	PE | 1x 1x | 10xA 1x | 10xB 10xA | 10xB	PE | 10xA PE | 10xB PE | 100x 1x | 100x 10xA | 100x 10xB | 100x
VA	PE | 1x 1x | 10xA 10xA | 10xB 10xB | 100x	PE | 10xA PE | 10xB PE | 100x 1x | 10xB 1x | 100x 10xA | 100x	PE | 1x 10xA | 10xB	PE | 10xA PE | 10xB PE | 100x 1x | 10xA 1x | 10xB 1x | 100x 10xA | 100x 10xB | 100x	PE | 1x PE | 10xB 1x | 10xA 1x | 10xB 10xA | 10xB 10xA | 100x 10xB | 100x	PE | 10xA PE | 100x 1x | 100x

Table S21. Summary of MIC data for the primary effluent during the antibiotic concentration testing phase.

Sample	Antibiotic	Sample Event	% Revived	% AR	Baseline MIC (µg/mL)	Observed MIC and Number of Isolates								Weighted Score	Avg. Score	St. Dev.	Avg. Score	St. Dev.
						<1x	1x	2x	4x	8x	16x	32x	>32x					
Primary Effluent	AMP	1	100%	100%	32	0	0	0	0	0	0	0	8	0	64.0	0.0	63.7	1.2
		2	88%	100%		0	0	0	0	0	0	0	7	0				
		3	100%	100%		0	0	0	0	0	0	0	8	0				
	SMX/TMP	1	100%	100%	76/4	0	0	0	0	0	0	1	7	0	62.7	2.3		
		2	100%	100%		0	0	0	0	0	0	0	8	0				
		3	88%	100%		0	0	0	0	0	0	0	7	0				
	TET	1	100%	100%	16	0	0	0	0	0	0	0	8	0	64.0	0.0		
		2	75%	100%		0	0	0	0	0	0	0	6	0				
		3	100%	100%		0	0	0	0	0	0	0	8	0				
	VA	1	100%	100%	4	0	0	0	0	0	0	0	8	0	64.0	0.0		
		2	100%	100%		0	0	0	0	0	0	0	8	0				
		3	88%	100%		0	0	0	0	0	0	0	7	0				

Table S22. Summary of MIC data for the 1x SBR during the antibiotic concentration testing phase.

Sample	Antibiotic	Sample Event	% Revived	% AR	Baseline MIC (µg/mL)	Observed MIC and Number of Isolates								Weighted Score	Avg. Score	St. Dev.	Avg. Score	St. Dev.
						<1x	1x	2x	4x	8x	16x	32x	>32x					
1x	AMP	1	88%	100%	32	0	0	0	0	0	0	0	7	64.0	61.0	5.2	61.3	5.0
		2	88%	86%		1	0	0	0	0	0	0	6	54.9				
		3	75%	100%		0	0	0	0	0	0	0	6	64.0				
	SMX/TMP	1	100%	100%	76/4	0	0	0	0	0	0	0	8	64.0	64.0	0.0		
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	100%	100%		0	0	0	0	0	0	0	8	64.0				
	TET	1	100%	100%	16	0	0	0	0	0	0	0	8	64.0	64.0	0.0		
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	100%	100%		0	0	0	0	0	0	0	8	64.0				
	VA	1	100%	88%	4	0	1	0	0	0	0	1	6	52.1	56.0	6.9		
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	100%	100%		0	0	0	0	0	2	0	6	52.0				

*Gray shading indicates isolate was inhibited by ≤1x MIC (i.e., not antibiotic resistant by definition)

Table S23. Summary of MIC data for the 10x(A) SBR during the antibiotic concentration testing phase.

Sam-ple	Antibiotic	Sample Event	% Revi-ved	% AR	Baseline MIC	Observed MIC and Number of Isolates								Weight-ed Score	Avg. Score	St. Dev.	Avg. Score	St. Dev.
					(µg/mL)	<1x	1x	2x	4x	8x	16x	32x	>32x					
10x (A)	AMP	1	100%	100%	32	0	0	0	0	0	0	0	8	64.0	64.0	0.0	61.3	6.3
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	100%	100%		0	0	0	0	0	0	0	8	64.0				
	SMX/TMP	1	100%	100%	76/4	0	0	0	0	0	0	0	8	64.0	64.0	0.0		
		2	88%	100%		0	0	0	0	0	0	0	7	64.0				
		3	100%	100%		0	0	0	0	0	0	0	8	64.0				
	TET	1	88%	100%	16	0	0	1	1	0	0	0	5	46.6	58.2	10.1		
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	100%	100%		0	0	0	0	0	0	0	8	64.0				
	VA	1	100%	100%	4	0	0	0	2	0	0	0	6	49.0	59.0	8.7		
		2	88%	100%		0	0	0	0	0	0	0	7	64.0				
		3	100%	100%		0	0	0	0	0	0	0	8	64.0				

Table S24. Summary of MIC data for the 10x(B) SBR during the antibiotic concentration testing phase.

Sam-ple	Antibiotic	Sample Event	% Revi-ved	% AR	Baseline MIC	Observed MIC and Number of Isolates								Weight-ed Score	Avg. Score	St. Dev.	Avg. Score	St. Dev.
					(µg/mL)	<1x	1x	2x	4x	8x	16x	32x	>32x					
10x (B)	AMP	1	100%	100%	32	0	0	0	0	0	0	0	8	64.0	56.5	13.0	61.7	6.5
		2	100%	88%		1	0	0	1	1	0	0	5	41.6				
		3	88%	100%		0	0	0	0	0	0	0	7	64.0				
	SMX/TMP	1	75%	100%	76/4	0	0	0	0	0	0	0	6	64.0	64.0	0.0		
		2	75%	100%		0	0	0	0	0	0	0	6	64.0				
		3	100%	100%		0	0	0	0	0	0	0	8	64.0				
	TET	1	100%	100%	16	0	0	0	0	0	0	0	8	64.0	64.0	0.0		
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	100%	100%		0	0	0	0	0	0	0	8	64.0				
	VA	1	100%	100%	4	0	0	0	0	0	0	0	8	64.0	62.5	2.6		
		2	88%	100%		0	0	0	0	0	0	1	6	59.4				
		3	100%	100%		0	0	0	0	0	0	0	8	64.0				

*Gray shading indicates isolate was inhibited by ≤1x MIC (i.e., not antibiotic resistant by definition)

Table S25. Summary of MIC data for the 100x SBR during the antibiotic concentration testing phase.

Sam-ple	Antibiotic	Sample Event	% Revi-ved	% AR	Baseline MIC	Observed MIC and Number of Isolates								Weight-ed Score	Avg. Score	St. Dev.	Avg. Score	St. Dev.
					(µg/mL)	<1x	1x	2x	4x	8x	16x	32x	>32x					
100x	AMP	1	75%	100%	32	0	0	0	0	0	0	0	6	64.0	64.0	0.0	61.5	4.9
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	88%	100%		0	0	0	0	0	0	0	7	64.0				
	SMX/TMP	1	75%	100%	76/4	0	0	0	0	0	0	0	6	64.0	61.4	4.5		
		2	100%	100%		0	0	1	0	0	0	0	7	56.3				
		3	100%	100%		0	0	0	0	0	0	0	8	64.0				
	TET	1	100%	100%	16	0	0	0	0	0	0	0	8	64.0	64.0	0.0		
		2	100%	100%		0	0	0	0	0	0	0	8	64.0				
		3	88%	100%		0	0	0	0	0	0	0	7	64.0				
	VA	1	100%	100%	4	0	0	1	1	0	0	0	6	48.8	56.6	7.6		
		2	100%	100%		0	0	0	0	1	0	0	7	57.0				
		3	100%	100%		0	0	0	0	0	0	0	8	64.0				

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