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Stay in the loop: lessons learned from studying the microbiological impacts of blending advanced-treated wastewater in pipe loops

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Supplementary Methods

Pipe loop rig design and start-up

The total water volume in each pipe loop was ~100 liters; this volume was selected to ensure sufficient microbial biomass could be concentrated from each pipe loop for genomic analyses; therefore, the 113-liter reservoir volume was selected based on this constraint. The pipe materials were chosen to be characteristic of the full-scale distribution system and premise plumbing, where the most common pipe categories are (in order of total length): cementitious, ferrous, plastic, copper, and brass. The pipe segments of copper, galvanized iron, leaded brass, and cement-lined ductile iron were each 7.6-cm in diameter and 1.5-m in length; the 12 removable PVC segments were each 2.5-cm in diameter and 0.3-m in length. The remaining PVC piping was 7.6-cm in diameter. Water was recirculated through the reservoir and piping at a flow rate of 5.05 x 10^{-4} m^3/s. This flow rate was chosen to target a fluid shear force of ~0.25 N/m^2 on the inner surfaces of the 0.3-m length removable pipe PVC segments for biofilm sampling. Fluid shear is the relevant scaling parameter with respect to biofilms and precipitates on piping surfaces, and an inner wall shear force of 0.25 N/m^2 is commonly described as characteristic of drinking water distribution systems.[1] However, the range in pipe diameters combined with the flow rate corresponds to a pipe wall shear stress ranging from 0.005 to 0.25 N/m^2 throughout the system. The total length of pipe was selected to ensure a sufficient fraction of the hydraulic residence time of the system was in the pipe loop itself as opposed to the reservoir. The design achieves this with a residence time of 1.9 minutes in the loop compared to 1.8 minutes in the reservoir while the water was recirculating. After the five pipe loops were constructed, two preparatory steps were carried out prior to initiation of the first study phase: (1) disinfection by high concentrations of free chlorine, and (2) inoculation with a drinking water microbial community collected from the full-scale conventional distribution system.

After construction, each pipe loop was disinfected prior to inoculation. Disinfection was carried out on October 16, 2017 by recirculating 100 liters of drinking water from a conventional drinking water treatment plant dosed to ~100 mg/L of free chlorine for 3 hours. Pipe loops were then drained and filled four times with 100 liters of drinking water from this source to diminish excess chlorine residual.

After disinfection, each pipe loop was inoculated with organisms concentrated from the full-scale drinking water distribution system. This inoculum was collected by dead-end ultrafiltration of drinking water from three public taps in the drinking water distribution system served by the conventional drinking water treatment facility over a 23-hour period. Prior to setting up the ultrafilters, the taps were flushed at max flow rate for ~10 minutes. In total, 6,070 L of tap water was concentrated by the three ultrafilters. Ultrafilters were backflushed into separate sterile bottles and the samples were held in the fridge for ~10 days before inoculation of the pipe loops. On the first day of pipe loop operation (October 16, 2017) the three bottles of inoculum were transported on ice to the pipe loop site, then homogenized by combining each bottle into a single sterile bottle and shaking vigorously for 10 s. Conventional drinking water was added to each of the pipe loops, and the inoculum was poured in equal volumes into each of the five pipe

loop reservoirs. The pipe loop pumps were turned on and run under standard operating conditions for seven days (recirculating the same water). Thereafter, the first day of Phase 1 operations began.

Short-term pipe loop operation protocols

There were two short-term protocols tested in Phase 1 that deviated from standard operations that were used in an attempt to stimulate biological growth. First, a 7-day recirculation period was employed for recirculation periods ending on November 13, 20, and 27, 2017. Second, for recirculation periods ending in early December, the total chlorine residual in the conventional feed water was intentionally decayed prior to recirculation by storing conventional feed water for 3.5 days.

In Phase 1, we attempted to lower the starting chlorine concentration in the conventional feedwater by intentionally degrading the residual in a small storage reservoir on-site. For recirculation periods ending on November 30 and December 4, 7, and 11, 2017, conventional feedwater was stored in the reservoir for ~3.5 days. Stored conventional feedwater was then chlorinated as per the procedure for the advanced feedwater. Thereafter, the intentional degradation of the chlorine residual was discontinued beginning on December 11, 2017.

Flow Cytometry

Two flow cytometers were used in this study as described in detail previously.[2] The Accuri was equipped with a 50 mW laser emitting a fixed wavelength of 488 nm, and measurements were performed at the “fast" flow rate of 66 µL minute^{-1} on sample volumes of 50 µL. Microbial cell signals were distinguished and enumerated from background and instrument noise on density plots of green (FL1; 533 ± 30 nm) and red (FL3; >670 nm) fluorescence using FlowJo gating software (v10.5.3). Gate positions were modified slightly from a template publicly available for the BD Accuri C6[3] to adapt for FlowJo software. The Canto was equipped with a 20 mW laser emitting a fixed wavelength of 488 nm, and measurements were performed at a flow rate of 1 µL s^{-1} for 50 seconds. Microbial cell signals were distinguished and enumerated from background and instrument noise on density plots of green (FTIC; 530 ± 30 nm) and red (PerCP; 695 ± 40 nm) fluorescence using FlowJo gating software. Gate positions were modified slightly compared to BD Accuri C6 gating based on calibration beads (Spherotech, Catalog # NFPPS-52-4K, Lake Forest, IL).

Biofilm Collection and Sampling

In a study of four methods for biofilm sampling from the interior surfaces of water pipes, Widmer and Jellison[4] reported the highest efficiencies in cell removal for scraping and sonication, and we combined both methods of biofilm recovery. Each end of a PVC segment for pipe loop biofilm recovery underwent a three-minute sonication with ~100 mL of 0.22 µm pore size-filtered water from the corresponding pipe loop. Next, the pipe segment was thoroughly scraped with a sterile cell scraper internally (catalog #RPI-162423CS; Research Products International, Mt. Prospect, IL), filled with 20 mL of filtered pipe loop bulk water, and shaken vigorously for 15 seconds. The cell scraper

was placed inside the shaken filtered pipe loop bulk water, and sonicated for 30 seconds. Sonicate from each step was combined to produce ~220 mL.

One pipe biofilm field blank was collected on January 18, 2018. The blank consisted of 225 mL of water from a pipe loop recirculating conventional drinking water that was filtered (0.22 µm) into a sterile bottle. The bottle was kept open during field sampling and underwent the laboratory sonication procedure. Finally, the water was concentrated using polyethylene glycol flocculation in parallel with field biofilm samples.

qPCR Methods

Amplification and quantification of qPCR targets were carried out in technical triplicate in MicroAmp™ Fast Optical 96-well optical plates (catalog #4346906, ThermoFisher Scientific) on a StepOnePlus™ Real-Time PCR System (software v2.3; Applied Biosystems, Foster City, CA). DNA standard curves on every plate consisted of 10-fold serial dilutions of gBlocks Gene Fragments (Integrated DNA Technologies, Coralville, IA) ranging from 10 to 10^9 gene copies, depending on assay, using PCR-grade water (catalog #AAJ60610-EQC, VWR, ThermoFisher Scientific) in DNA LoBind® 0.5 mL (catalog #22431005, Eppendorf®, Millipore Sigma) or 5 mL tubes (catalog #Z768820-200EA, Sigma Aldrich) (Table S5). Triplicate negative controls (i.e., PCR-grade water) were run on every plate. gBlock™ standards were prepared as follows: probes were prepared as 100 nm PrimeTime 5' 6-FAM/ZEN/3' IBFQ (16S rRNA) or PrimeTime Eco 5' 6-FAM/ZEN/3' IBFQ (*Legionella pneumophila*) purified by HPLC. Primers for all assays were prepared as gBlocks™ Gene Fragments, RxnReady® Primer Pool – Oligo Mix Products purified by standard desalting. On the StepOnePlus software, we applied the same threshold to all samples within an assay. The threshold was selected based on average threshold values determined by the instrument and checked visually to best cross the linear portions of every standard and sample amplification curve. Table S5 shows the thresholds chosen for each assay.

Data analysis of qPCR results was completed in R (v4.1.3). The limit of quantification (LoQ) for each assay was experimentally determined as the lowest concentration on each standard curve that was at least three standard deviations higher than the mean concentration of the negative control and for which at least 75% of triplicates amplified. The LoQs were determined to be 1,000 gene copies per reaction for the 16S rRNA gene and 10 genes copies per PCR reaction for all other qPCR assays. The negative controls for the 16S rRNA gene amplified but below the LoQ (i.e., not within the linear region of the standard curves). Negative controls and field blanks for all other assays did not amplify.

Thermal cycling conditions for each assay were based on previous studies (Table S9) and optimized before analysis of any samples. Melt curves (SYBR Green™ chemistry assays) were used to evaluate non-target amplification and confirm amplification of target DNA (results not shown). Inhibition testing of samples (Table S10) followed the spike and dilute method to determine possible inhibition of qPCR assays by interfering substances in the water samples and subsequent need for sample dilution.[5] Based on

inhibition testing results, sample DNA was diluted as necessary to ensure <100 ng of DNA was added to each well.

Reactions (total volume: 20 µL) were performed manually in triplicate with purified sample DNA (5 µL) and reaction mix (15 µL). Assays utilized either TaqMan™ Environmental Master Mix 2.0 chemistry (catalog #4396838, ThermoFisher Scientific) or PowerUp™ SYBR™ Green Master Mix (catalog #A25780, ThermoFisher Scientific). Each reaction mix (Table S9) consisted of master mix (10 µL), bovine serum albumen to minimize potential inhibition (0.3 µM; catalog #15260037, ThermoFisher Scientific), primers and probes (Table S8), and nuclease-free water to yield 15 µL. To determine if samples were inhibited for PCR, a subset of samples was selected for PCR inhibition testing. No samples were inhibited (Table S10); therefore, no dilution was used for some samples. However, sample DNA was diluted as necessary to retain sufficient sample DNA to complete all intended analyses (e.g., multiple qPCR assays and genomic sequencing) and to ensure less than 100 ng of DNA was added to each qPCR plate well. Sample dilutions varied from no dilution to 100-fold dilution. Of the 242 samples analyzed among all of the qPCR assays, one sample was diluted 100-fold, four samples were diluted 20-fold, and 16 samples were diluted 10-fold; all other samples were diluted 5-fold or less.

Supplementary Tables

Table S1: Summary of physical and chemical water quality analyses applied to feed waters and pipe loops.

Parameter	Frequency of Sampling	Location of Analysis	Analytical Method	Method Reference
pH	Daily	Field	pH probe	SM 4500-H^+
Temperature	Daily	Field	Thermometer	
Total Chlorine	Daily	Field	Spectrophotometric	SM 4500-Cl G
Free Chlorine	Twice Weekly	Field	Spectrophotometric	SM 4500-Cl G
Alkalinity	Weekly	Lab		SM 2320 B 1997
Major Anions[2]: Cl^-, SO_4^{2-}, F^-, NO_3^-, CO_3^{2-}, PO_4^{3-}	Weekly	Lab	Ion Chromatography	EPA 300.0; SM 2320 B 1997 (CO_3^{2-} only)
Major Cations: Ca^{2+}, Mg^{2+}, Na^+, K^+	Weekly	Lab	Atomic Absorption Spectrometry	SM 3111 B 1999
Metals: Lead and Copper	Weekly	Lab	Inductively Coupled Plasma Mass Spectrome	EPA 200.8

Table S2: Microbial assays completed for bulk water and biofilm samples.

Target Group	Parameter / Target	Bulk Water	Bio-film	Description and Rationale
Microbial Abundance and Viability	Total cell count by flow cytometry	X		Quantify microbial cells[6,7]
	Intact cell count by flow cytometry	X		Quantify microbial cells with intact membranes[7,8]
	Total ATP	X	X	Quantify microbial biomass and assess microbial viability[8]
	Intracellular ATP (or microbial ATP)	X	X	
	16S rRNA Gene	X	X	Quantify bacteria (16S rRNA gene)[9]; Calculate relative abundance for other targets (*blaTEM* and *sul1*)
Oppor-tunistic pathogens	*Legionella pneumophila*	X	X	Quantify opportunistic pathogens that are the leading causes of drinking water-related illness in th USA; frequently detected in biofilm and bulk water c drinking water distribution systems[10]
	Mycobacterium avium Complex (MAC)	X	X	
	Acanthamoeba spp.	X	X	Quantify free-living parasitic amoeba; host for *L. pneumophila* and MAC[11]
Antibiotic resistance genes	bla_{TEM}	X	X	Quantify emerging microbial contaminants with potential public health impacts.[12] Bla_{TEM}[13] and *sul1*[14] frequently detected in wastewater and are proposed indicators for antibiotic resistance in wastewater systems
	sul1	X	X	
Microbial Community Profile	16S rRNA gene (V4 region)	X	X	Examine changes in microbial community profile[15,16] and identify targets to quantify with qPCR

Table S3: Total chlorine data for pipe loops at the end of recirculation periods in Phases 1 and 2, where n is the total number of samples for which total chlorine concentrations were determined.

Pipe loop	Phase	n	Average total chlorine concentration (mg/L as Cl_2)	Samples < 0.02 mg/L as Cl_2 (%)	Samples < 0.2 mg/L as Cl_2 (%)
Loop 1	Phase 1	18	0.096	16.7	83.3
Loop 1	Phase 2	22	0.11	9.09	86.4
Loop 2	Phase 1	18	0.26	5.56	44.4
Loop 2	Phase 2	22	0.20	4.55	68.2
Loop 3	Phase 1	18	0.56	0	5.56
Loop 3	Phase 2	22	0.18	0	68.2
Loop 4	Phase 1	18	0.50	0	22.2
Loop 4	Phase 2	22	0.21	0	63.6
Loop 5	Phase 1	18	0.26	0	50
Loop 5	Phase 2	22	0.79	0	0

Table S4: Physical and chemical water quality results from the pipe loops and feed waters. Mean values by phase (Phases 1 and 2) are shown. Alk = alkalinity. When data were not collected, the box is marked as “NC”.

Phase	Sample Type	Temp	pH	Alk	Ca^{2+}	Cu^{2+}	Mg^{2+}	Pb^{2+}	NO_3-N^-	PO_4^{3-}	SO_4^{2-}	Cl^-	F^-	K^+	Na^+
	units	°C	-	mg/L as $CaCO_3$	mg/L	µg/L	mg/L	µg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L	mg/L
1	Conventional feedwater	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
1	Advanced feedwater	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC	NC
1	Pipe loops fed with 100% conventional feedwater	18	8.3	93	39	15	15	4.19	0.31	<0.05	87	73	0.53	4.86	59
1	Pipe loops fed with the advanced blend	18	8.6	100	32	38	5	8.24	1.93	<0.05	23	32	0.14	3.35	35
2	Conventional feedwater	16	8.1	85	33	7	15	<0.3	0.34	<0.05	88	76	0.53	3.08	62
2	Advanced feedwater	17	8.1	101	38	2	4	<0.3	1.58	0.22	12	24	<0.05	1.55	33
2	Pipe loops fed with 100% conventional feedwater	15	8.3	86	34	31	14	5.8	0.3	0.05	80	73	0.52	3.42	76
2	Pipe loops fed with the advanced blend	15	8.4	92	34	122	5	10.0	1.68	0.06	25	33	0.15	1.99	32

Table S5: Summary statistics for qPCR standard curves, including amplification efficiency, R^2, and number of replicates that amplified for the lowest quantity of the qPCR standard curves.

	16S rRNA Gene	***Legionella pneumophila***	***Mycobacterium avium* complex**	***Acanthamoeba* spp.**	***bla*$_{TEM}$**	***sul1***
# of Standard Curves	10	5	6	5	7	6
Threshold applied	0.14	1	0.25	0.23	0.4	0.4
Linear range (gene copies per qPCR well)	10^3 - 10^9	10^1 - 10^7	10^1 - 10^6	10^1 - 10^6	10^1 - 10^6	10^1 - 10^6
Amplification Efficiency						
Arithmetic mean	84.4	93.2	83.3	85.2	81.2	83.8
Standard deviation	7.0	3.8	6.2	3.7	3.9	6.6
Maximum	93.6	98.1	89.6	90.3	87.7	92.7
Minimum	73.3	88.4	76.2	80.0	77.3	73.9
R^2 Values						
Mean	0.996	0.998	0.998	0.993	0.998	0.998
St. Dev.	0.004	0.001	0.001	0.002	0.001	0.001
# of Replicates Amplifying at Low End of Curve (i.e., 1,000 copies for 16S, 10 copies for all other assays)						
Arithmetic mean	3	3	2.3	2.3	2.4	2.7
Minimum	3	3	1	1	2	2
Maximum	3	3	3	3	3	3
% of All Replicates that Amplified	100	100	78	78	81	89

Table S6: qPCR standard curve information for each plate. Each plate had one standard curve run on it with three replicates at each 10-fold dilution step of the standard curve. None of the qPCR negative controls for 16S rRNA gene amplified within the linear range of the standard curve. *LoQ = limit of quantification as defined in the main manuscript methods. **qPCR negative controls were PCR-grade water as defined in the methods. ***Standard was accidentally not added to the 1,000 gene copy wells (the LoQ) for the 16S rRNA gene standard curve on 3/9/19 and a master standard curve of all other 16S standard curves was used to convert Cq to quantities. *Mycobacterium avium* complex is abbreviated as "MAC."

Plate #	Date Run	Assay	Effic-iency	Slope	Y-intercept	R^2	Number of Standard Curve Replicates that Amplified at the LoQ*	Number of qPCR Negative Control** Replicates that Amplified	Avg C_q at LoQ	Standard Deviation of C_q at LoQ
101	2/9/19	acantha-moeba	82.04	-3.844	41.797	0.994	2	0	37.46	0.80
103	2/9/19	MAC	87.94	-3.649	41.063	0.998	3	0	37.21	0.41
104	2/9/19	legionella	94.77	-3.454	41.716	0.999	3	0	38.46	0.34
105	2/9/19	blatem	87.65	-3.658	40.425	0.999	3	0	36.78	0.25
106	2/18/19	sul1	92.65	-3.511	40.214	0.998	3	0	36.75	0.57
107	2/18/19	S16	91.05	-3.557	41.728	0.999	3	3	31.22	0.06
108	2/18/19	S16	84.9	-3.746	42.534	0.998	3	3	31.30	0.26
109	2/18/19	acantha-moeba	90.34	-3.577	39.921	0.992	3	0	36.53	1.17
111	2/20/19	MAC	76.23	-4.064	42.952	0.997	1	0	38.58	NA
112	2/21/19	legionella	98.09	-3.369	41.259	0.998	3	0	38.08	0.85
113	2/21/19	sul1	87.13	-3.674	40.727	0.998	3	0	37.33	0.68
114	2/21/19	blatem	77.36	-4.018	42.848	0.998	2	0	38.39	0.46
115	2/24/19	S16	88.33	-3.637	41.518	0.998	3	3	30.69	0.08
116	2/24/19	acantha-moeba	85.23	-3.736	40.229	0.992	3	0	36.81	1.08
118	2/24/19	MAC	88.95	-3.619	40.077	0.999	3	0	36.41	0.37
119	2/24/19	legionella	94.25	-3.468	41.5	0.999	3	0	38.15	0.40

120	2/26/19	sul1	82.47	-3.829	42.021	1	2	0	37.96	0.10
121	2/26/19	blatem	79.6	-3.932	42.741	0.998	2	0	38.32	0.74
122	2/26/19	S16	78.48	-3.975	44.065	0.996	3	3	32.85	0.16
123	2/26/19	acantha-moeba	86.91	-3.681	40.776	0.992	2	0	36.58	1.24
124	2/26/19	MAC	77.98	-3.994	42.511	0.997	2	0	37.87	0.61
125	2/27/19	sul1	77.06	-4.03	42.92	0.998	2	0	39.17	1.18
126	3/5/19	blatem	80.49	-3.899	42.048	0.996	3	0	37.94	1.04
127	3/5/19	legionella	90.36	-3.577	42.504	0.999	3	0	38.83	0.58
128	3/6/19	legionella	88.4	-3.635	42.752	0.997	3	0	39.25	1.12
129	3/9/19	16S	78	-3.994	44.807	0.992	3	3	***	***
130	3/7/19	sul1	88.49	-3.632	39.872	0.999	3	0	36.05	0.22
131	3/7/19	blatem	80.8	-3.889	41.534	0.997	3	0	38.12	0.67
132	3/12/19	acantha-moeba	86.94	-3.681	40.256	0.997	3	0	36.92	0.28
134	3/9/19	MAC	89.6	-3.599	40.465	0.997	3	0	36.87	0.80
136	3/12/19	MAC	78.85	-3.96	42.177	0.998	2	0	38.12	0.46
137	3/20/19	S16	77.72	-3.979	45.24	0.994	3	3	33.74	0.06
138	3/12/19	blatem	85.198	-3.736	41.7	0.997	2	0	37.44	0.35
139	3/19/19	sul1	85.034	-3.742	40.899	0.997	3	0	37.44	0.97
140	3/19/19	S16	93.555	-3.487	41.638	0.997	3	3	31.38	0.09
144	3/26/19	blatem	77.3	-4.02	43.184	0.998	2	0	39.16	1.33
145	3/27/19	S16	90.623	-3.569	43.611	0.987	3	3	33.17	0.41
148	3/28/19	S16	73.276	-4.189	51.799	0.996	3	3	39.21	0.63
151	7/1/19	S16	87.648	-3.658	42.556	1	3	3	31.68	0.08

Table S7: Summary table of gBlocks™ standards.

	Standard A	Standard B	Standard C
Includes F and R Primers for what Targets?	16S rRNA Gene, Legionella pneumophila	Acanthamoeba spp., sul1, Mycobacterium avium complex	bla_{tem}
Length	740	997	748
Melting temp (degrees Celsius)	80.6	80.4	78.1
GC Content	0.5243	0.5145	0.4626
Pass IDT complexity screening?	yes	yes	yes
Primer sequences in standard verified?	yes	yes	yes

Table S8: Forward (F) and reverse (R) primers and probe sequences for qPCR assays.

Gene Target	Primer or Probe	Target Name	Target Sequence (5' --> 3')	Ref
16S rRNA gene	F Primer	16SUni-F (900)	TCCTACGGGAGGCAGCAGT	9
	R Primer	16SUni-R (900)	GGACTACCAGGGTATCTAATCCTGTT	
	Probe	16SUni-P (250)	6FAM-CGTATTACCGCGGCTGCTGGCAC-TAMRA	
Legionella pneumophila	F Primer	LmipF:	AAAGGCATGCAAGACGCTATG	17
	R Primer	LmipR:	GAAACTTGTTAAGAACGTCTTTCATTTG	
	Probe	Probe:	FAM-TGGCGCTCAATTGGCTTTAACCGA-TAMRA	
***Mycobacterium avium* complex**	F Primer	MACF	CCCTGAGACAACACTCGGTC	18
	R Primer	MACR	ATTACACATTTCGATGAACGC	
Acanthamoeba spp.	F Primer	AcantF900	CCCAGATCGTTTACCGTGAA	19
	R Primer	AcantR1100	TAAATATTAATGCCCCCAACTATCC	
***bla*$_{TEM}$**	F Primer	bla-TEM, FX	GCKGCCAACTTACTTCTGACAACG	20
	R Primer	bla-TEM, RX	CTTTATCCGCCTCCATCCAGTCTA	
sul1	F Primer	sul1-FW	CGCACCGGAAACATCGCTGCAC	20
	R Primer	sul1-RV	TGAAGTTCCGCCGCAAGGCTCG	

Table S9: Reaction mixes and thermal cycling conditions for qPCR assays.

Gene Target	Ref	Reaction Mix	Reaction Conc. (µM)	Reaction Cycling	Temp (ºC)	Time (sec)
16S rRNA gene	9	TaqMan™ Environmental 2.0	(1x)	Pre-denaturation for 95C for 10 min		
		Primer (F and R)	0.9	Denaturation	95	15 s
		Probe	0.25	Annealing	60	30 s
		Bovine serum albumen	0.05	Extension	72	60 s
Legionella pneumophila	17	TaqMan Environmental 2.0	(1x)	Pre-denaturation for 95C for 10 min		
		Primer (F and R)	0.9	Denaturation	95	15 s
		Probe	0.25	Annealing	60	60 s
		Bovine serum albumen	0.05			
***Mycobacterium avium* complex**	18	PowerUp SYBR Green	(1x)	UDG activation for 2 min + Pre-denaturation for 95C for 5 min		
		Primer (F and R)	0.35	Denaturation	95	15 s
		Probe	NA	Annealing	55	30 s
		Bovine serum albumen	0.05	Extension	72	60 s
***Acanthamoeba* spp.**	19	PowerUp SYBR Green	(1x)	UDG activation for 2 min + Pre-denaturation for 95C for 1 min		
		Primer (F and R)	0.25	Denaturation	95	15 s
		Probe	NA	Annealing	60	60 s
		Bovine serum albumen	0.05	Extension	72	60 s
blaTEM	20	PowerUp™ SYBR™ Green	(1x)	UDG activation for 2 min + Pre-denaturation for 95C for 3 min		
		Primer (F and R)	0.3	Denaturation	95	15 s
		Probe	NA	Annealing	60	20 s
		Bovine serum albumen	0.05	Extension	72	60 s

sul1	20	PowerUp™ SYBR™ Green	(1x)	UDG activation for 2 min + Pre-denaturation for 95C for 3 min		
		Primer (F and R)	0.3	Denaturation	95	15 s
		Probe	NA	Annealing	60	30 s
		Bovine serum albumen	0.05	Extension	72	60 s

Table S10: qPCR inhibition testing results. Samples were deemed “not inhibited” when the difference between measured delta(Ct) and expected delta(Ct) (see the right-most column) wass less than one. Sample dilutions that meet criteria for “not inhibited” are shaded green in the right column. *Mycobacterium avium* complex is abbreviated as “MAC.” Note that the header rows repeat on each new page.

		Expected delta(Ct) between dilution			Measured Ct at Specified Dilution Factor (x1, 2, 5, 10)				Difference Between Measured delta(Ct) and Expected delta(Ct)		
		Dilution Factor									
Assay	**Sample Type**	**x2**	**x5**	**x10**	**x1**	**x2**	**x5**	**x10**	**x2**	**x5**	**x10**
Acantha-moeba	Pipe loop fed with 100% conventional feedwater (bulk water)										
	Pipe loop fed with t advanced blend (bulk water)	1.16			31.40	32.70			0.14		
	Pipe loop fed with 100% conventional feedwater (biofilm)		1.36	2.40		27.76	29.70	30.73		0.58	0.57
	Pipe loop fed with t advanced blend (biofilm)	1.16			31.50	32.30			-0.36		
	Conventional feedwater		1.36	2.40		27.98	30.01	31.10		0.67	0.72
	Advanced feedwater	1.03	2.40	3.43							
***bla*$_{TEM}$**	Pipe loop fed with 100% conventional feedwater (bulk water)		1.54	2.71		28.41	30.18	32.02		0.23	0.90
	Pipe loop fed with t advanced blend (bulk water)	1.04			29.58	30.78			0.16		
	Pipe loop fed with 100% conventional feedwater (biofilm)		1.54	2.71		28.40	30.48	31.73		0.54	0.62
	Pipe loop fed with t advanced blend (biofilm)	1.04			29.63	31.05			0.38		
	Conventional feedwater	1.17	2.71	3.88		28.51	30.44	31.49		-0.78	-0.90
	Advanced feedwater		1.54	2.71		28.32	30.25	31.36		0.39	0.33

		Expected delta(Ct) between dilution			Measured Ct at Specified Dilution Factor (x1, 2, 5, 10)				Difference Between Measured delta(Ct) and Expected delta(Ct)		
		Dilution Factor									
Assay	**Sample Type**	**x2**	**x5**	**x10**	**x1**	**x2**	**x5**	**x10**	**x2**	**x5**	**x10**
MAC	Pipe loop fed with 100% conventional feedwater (bulk water)		1.50	2.64		24.56	26.45	27.70		0.39	0.50
	Pipe loop fed with t advanced blend (bulk water)	1.10			30.81	31.77			-0.14		
	Pipe loop fed with 100% conventional feedwater (biofilm)		1.50	2.64		24.50	25.95	27.00		-0.05	-0.14
	Pipe loop fed with t advanced blend (biofilm)	1.10			31.03	31.82			-0.31		
	Conventional feedwater		1.50	2.64		24.73	26.65	27.97		0.42	0.60
	Advanced feedwater		1.50	2.64		24.58	26.57	27.63		0.49	0.41
sul1	Pipe loop fed with 100% conventional feedwater (bulk water)	1.14	2.65	3.80	29.67	31.58	23.65		0.77		
	Pipe loop fed with t advanced blend (bulk water)	1.14	2.65	3.80	29.54	30.97	31.29	32.70	0.29	-0.90	-0.64
	Pipe loop fed with 100% conventional feedwater (biofilm)	1.14	2.65	3.80	28.63	29.72	31.66	32.84	-0.05	0.38	0.41
	Pipe loop fed with t advanced blend (biofilm)	1.14	2.65	3.80	30.40	31.90			0.36		
	Conventional feedwater	1.14	2.65	3.80	29.72	31.50	33.90		0.64	1.53	
	Advanced feedwater	1.14	2.65	3.80	19.73	20.74	22.58	23.65	-0.13	0.20	0.12
16S rRNA Gene	Pipe loop fed with 100% conventional feedwater (bulk water)	1.12	2.61	3.73	21.98	22.75	24.06	25.14	-0.35	-1.30	-2.65

		Expected delta(Ct) between dilution			Measured Ct at Specified Dilution Factor (x1, 2, 5, 10)				Difference Between Measured delta(Ct) and Expected delta(Ct)		
		Dilution Factor									
Assay	**Sample Type**	**x2**	**x5**	**x10**	**x1**	**x2**	**x5**	**x10**	**x2**	**x5**	**x10**
	Pipe loop fed with t advanced blend (bulk water)	1.12	2.61	3.73	21.38	22.40	23.64	24.70	-0.10	-1.37	-2.67
	Pipe loop fed with 100% conventional feedwater (biofilm)	1.12	2.61	3.73	21.22	21.73	24.80	26.00	-0.61	0.46	-2.53
	Pipe loop fed with t advanced blend (biofilm)	1.12	2.61	3.73	20.73	21.73	23.05	24.21	-0.12	-1.29	-2.57
	Full-scale distribution system	1.12	2.61	3.73	20.00	21.01	22.42	23.56	-0.11	-1.20	-2.59
	Advanced feedwater	1.12	2.61	3.73	16.91	17.85	19.08	20.24	-0.18	-1.38	-2.57
Legionella	Pipe loop fed with 100% conventional feedwater (bulk water)		1.56	2.75		29.53	31.50	32.80		0.41	0.52
	Pipe loop fed with t advanced blend (bulk water)	1.04			32.56	33.56			-0.04		
	Pipe loop fed with 100% conventional feedwater (biofilm)		1.56	2.75		29.53	31.50	32.80		0.41	0.52
	Pipe loop fed with t advanced blend (biofilm)	1.04			32.50	33.51			-0.03		
	Conventional feedwater		1.56	2.75		29.53	31.50	32.80		0.41	0.52
	Advanced feedwater		1.56	2.75		29.53	31.50	32.80		0.41	0.52

Table S11: The RO permeate used in the study was from an advanced treatment facility at a different location than the pipe loops, and batch growth assays under carbon-limiting conditions were used to determine if carbon was introduced during RO handling. A grab sample was collected at each step of RO permeate handling, including conveyance, transportation, and storage ("Sample Location"). The total cell count at the start of the assay ("Start") was compared to that after 5 days of incubation ("End"). The absolute increase and the fold increase in cells are shown throughout RO handling and also in the experimental control for comparison.

	Total Cell Counts (cells/mL)			
Sample Location	**Start T = 0 days**	**End T = 5 days**	**Absolute Increase**	**Fold Increase**
RO permeate	$10^{3.66}$	$10^{3.72}$	$10^{2.79}$	1.1
RO permeate storage tank (Onsite at advanced wastewate treatment facility)	$10^{4.14}$	$10^{4.74}$	$10^{4.62}$	4.0
RO permeate conveyance (Feedline to truck)	$10^{4.40}$	$10^{4.71}$	$10^{4.40}$	2.0
Truck effluent	$10^{4.49}$	$10^{5.43}$	$10^{5.38}$	8.8
Stored, conditioned RO permeate	$10^{5.49}$	$10^{5.63}$	$10^{5.09}$	1.4
Experimental control (Bottled mineral Evian water)	$10^{4.94}$	$10^{5.01}$	$10^{4.17}$	1.2

Supplementary Figures

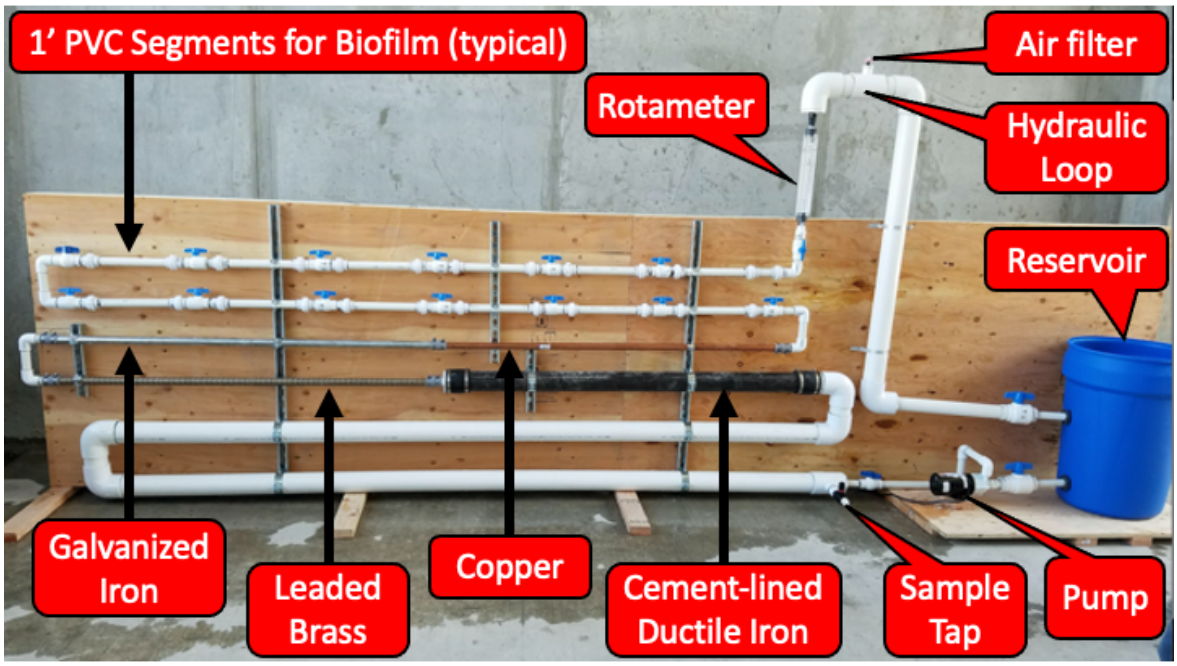

Figure S1: A constructed pipe loop rig with components labeled. Unlabeled piping is made of PVC. The reservoir was covered with a lid during operation (not shown).

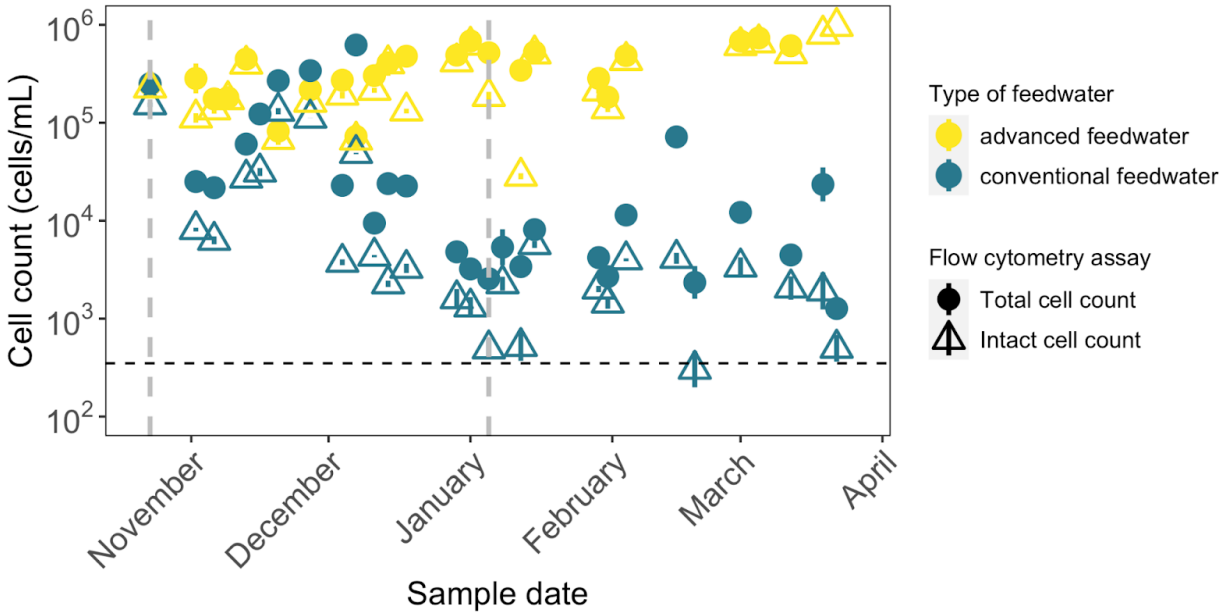

Figure S2: Total and intact cell counts by flow cytometry in the conditioned RO permeate and conventional feedwater over the study period. The horizontal dashed line represents the geometric mean total cell count in RO permeate (~350 cells/mL). The vertical gray dashed lines denote phase changes (from left-to-right: inoculation → Phase 1 → Phase 2). Error bars indicate geometric standard deviation of technical triplicates; the absence of an error bar indicates that error is smaller than the marker.

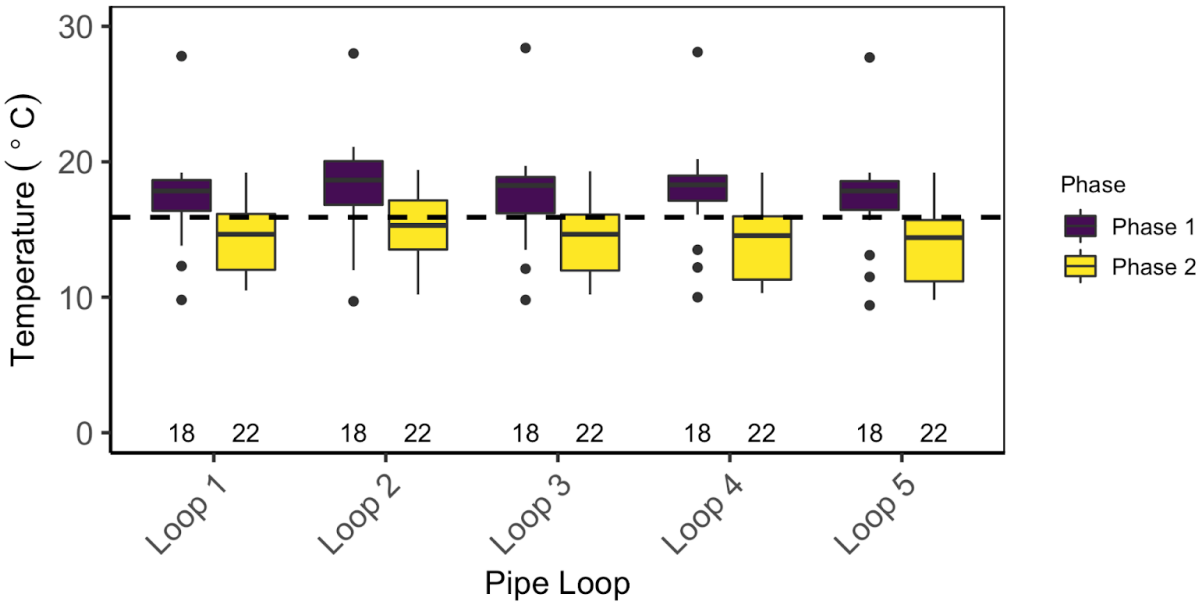

Figure S3: Boxplot of temperature in the pipe loops at the end of each recirculation period by study phase. Horizontal dashed line denotes the mean temperature of the pipe loops (15.9 °C) for all phases. The total number of samples taken for each sample group is located immediately above the x-axis.

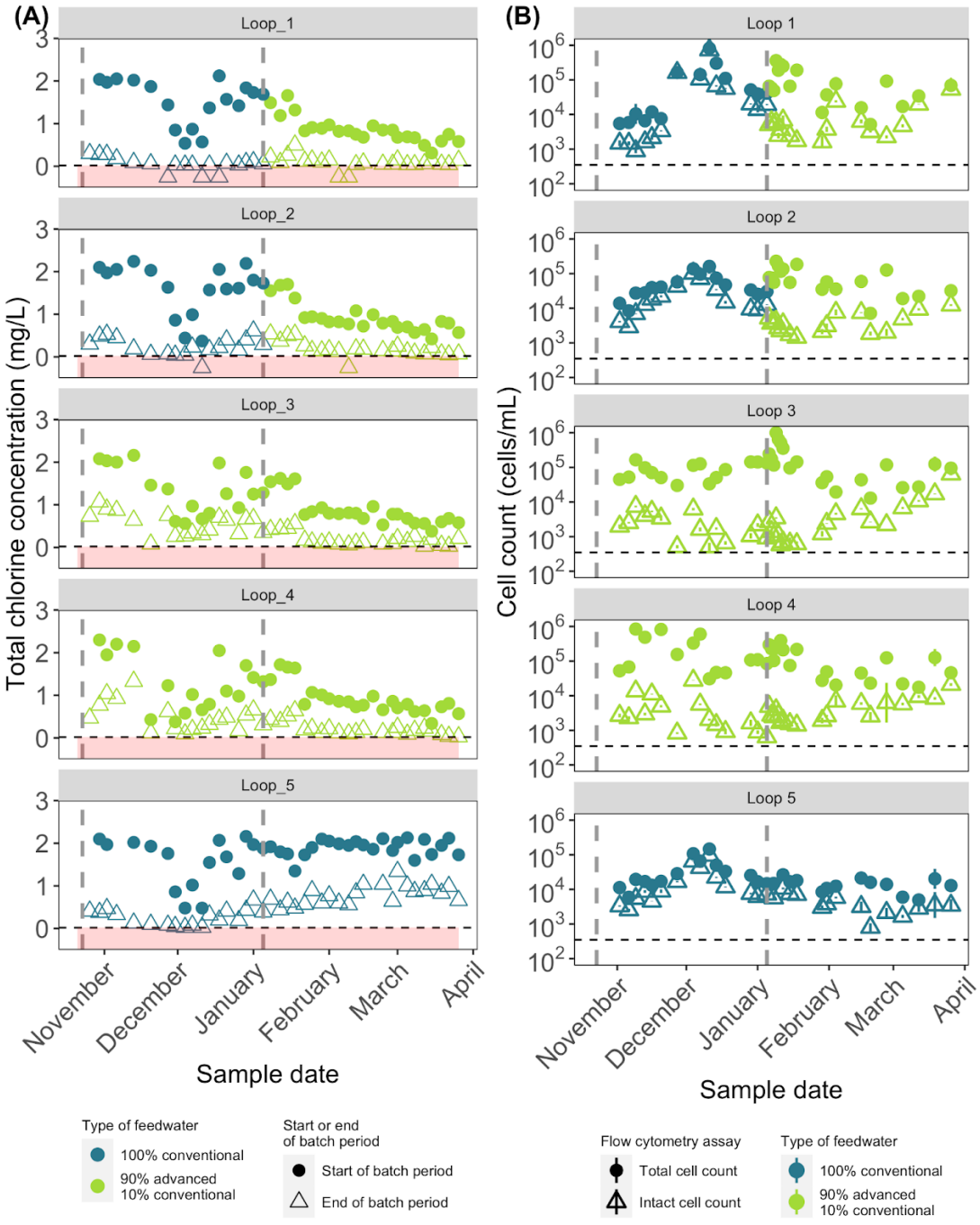

Figure S4: (A) Total chlorine concentration in the pipe loops at the start and end of recirculation periods, where the red area (lightly shaded) below the horizontal dashed line illustrates <0.02 mg/L of total chlorine, and total chlorine values that were <0.02 mg/L are plotted below the dashed line for visualization. The decrease in chlorine concentration (particularly in loops 1, 2, and 5) in late November/ early December are from a short term recirculation protocol to decrease chlorine concentration of the conventional feedwater. (B) Total and intact cell counts by flow cytometry in the pipe loops over the study period. The horizontal dashed line represents the geometric mean total cell count in RO permeate

(~350 cells/mL). The vertical gray dashed lines denote phase changes (from left-to-right: inoculation → Phase 1 → Phase 2). Time series from individual pipe loop experimental replicates are shown including the transition loops (“Loop 1” and “Loop 2”), the advanced blend loops (“Loop 3” and “Loop 4”) and the conventional control loop (“Loop 5”).

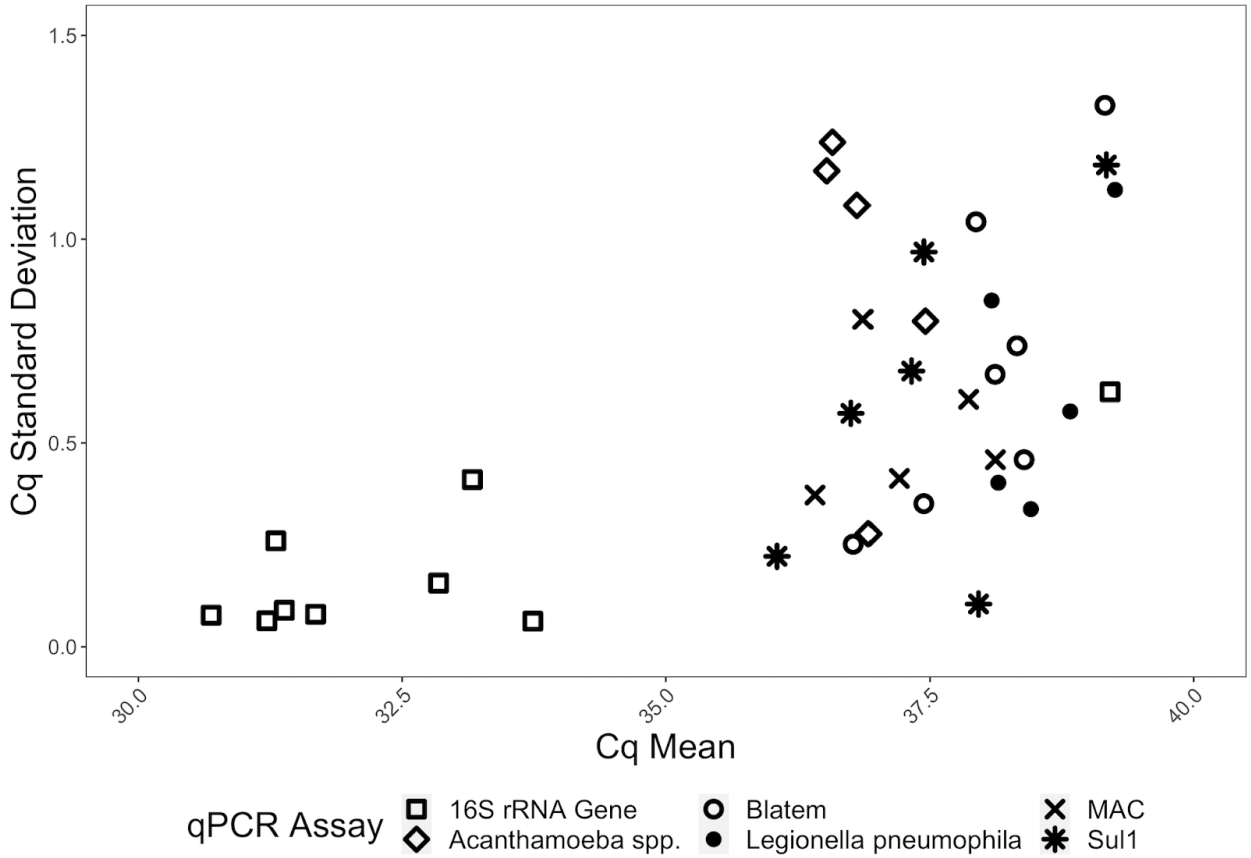

Figure S5: Cq Mean versus standard deviation for standard curve replicates at the respective standard curve LoQ (i.e., 1,000 gene copies per well for the 16S rRNA gene, and 10 gene copies per well for all other qPCR assays).

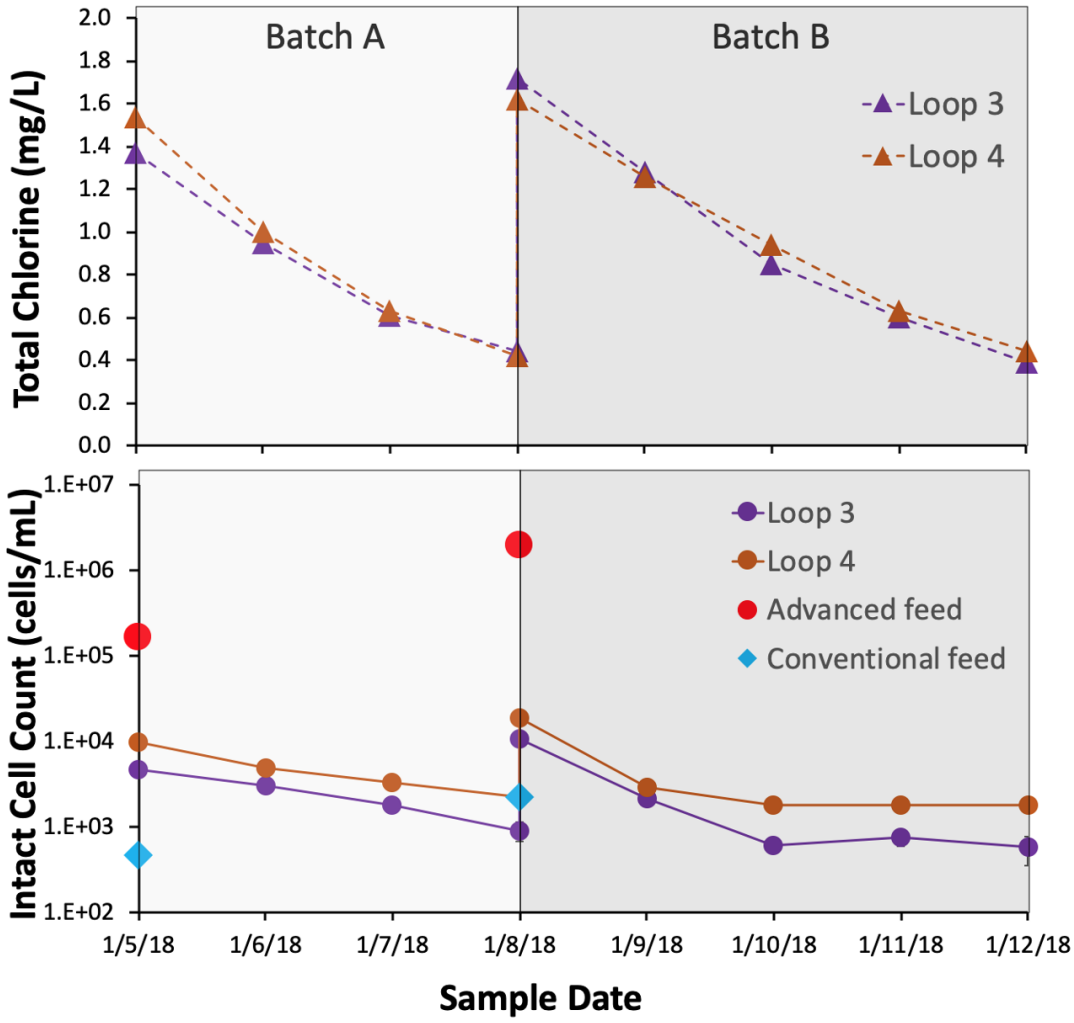

Figure S6: Total chlorine concentration and intact cell counts in feed waters and in the advanced blend loops (Loops 3 & 4) over the first two recirculation periods of Phase 2. Error bars indicate geometric standard deviation of technical triplicates; the absence of error bars indicates that error is smaller than the marker.

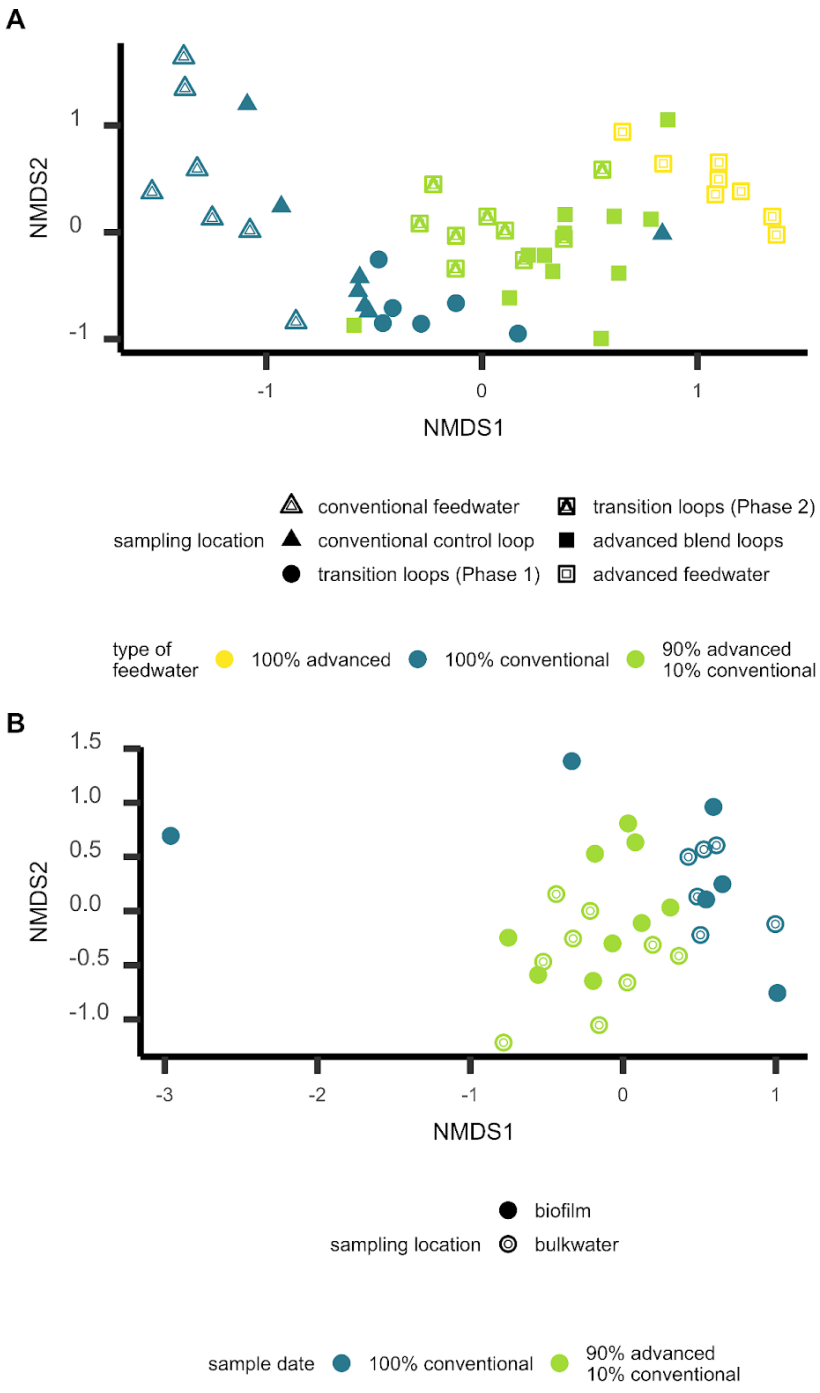

Figure S7: Non-metric multidimensional scaling of Bray-Curtis dissimilarity for (A) pipe loop bulk water microbial communities via 16S rRNA gene sequencing (stress= 0.15) and (B) bulk water and biofilm samples from transition loop samples (stress= 0.18)

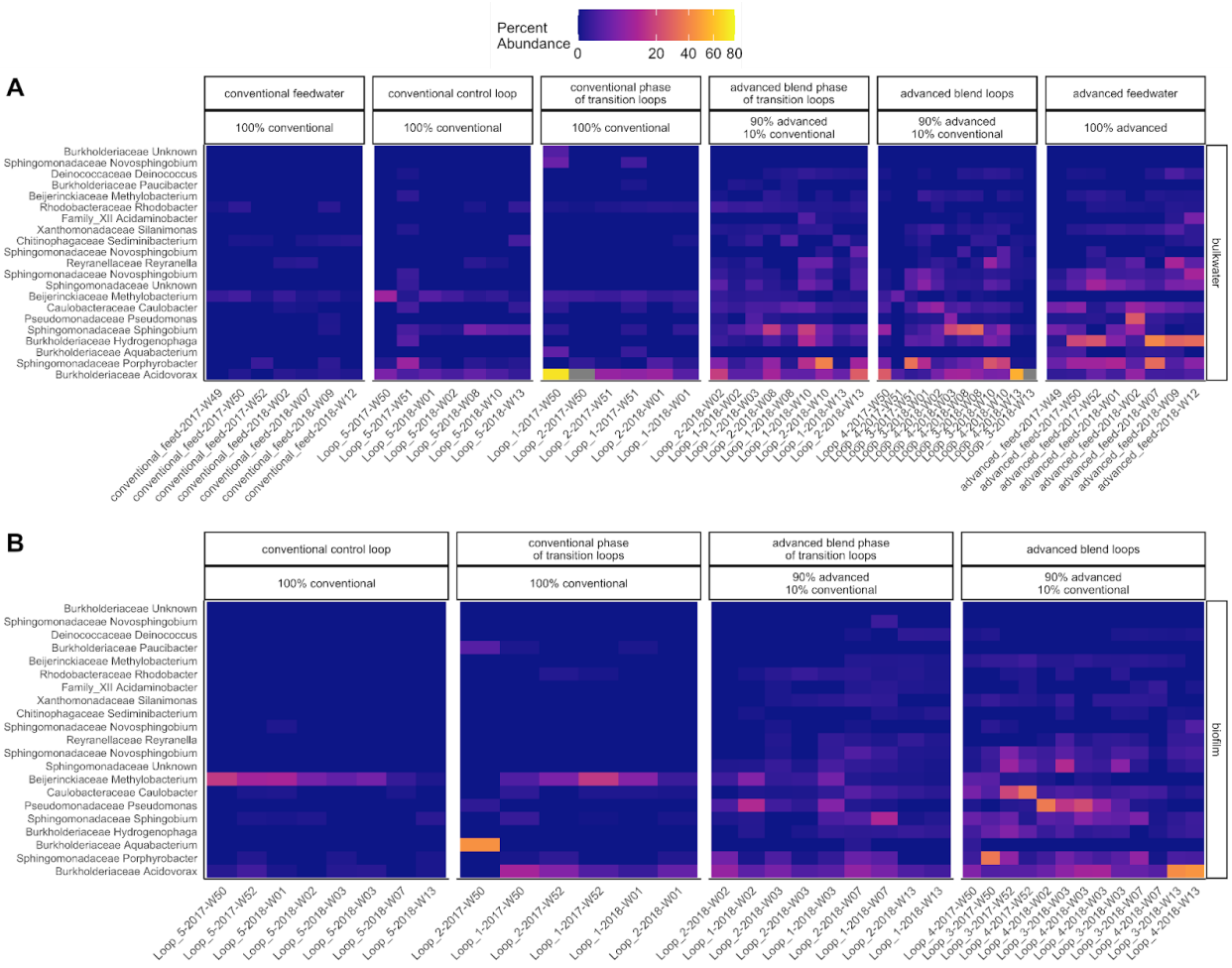

Figure S8: Heatmap of bulk water (A) and biofilm (B) percent abundance (see scale at the top of the figure) of ASVs identified to significantly change in the transition loops in Phase 2 (rows) by sample type (column facet) across sampling dates (columns - in the format Loop_x-yyyy-Wzz, where x is the pipe loop number (1-5), yyyy is the year, and zz is a number from 01-52 that indicates which week of the year the sample was collected)

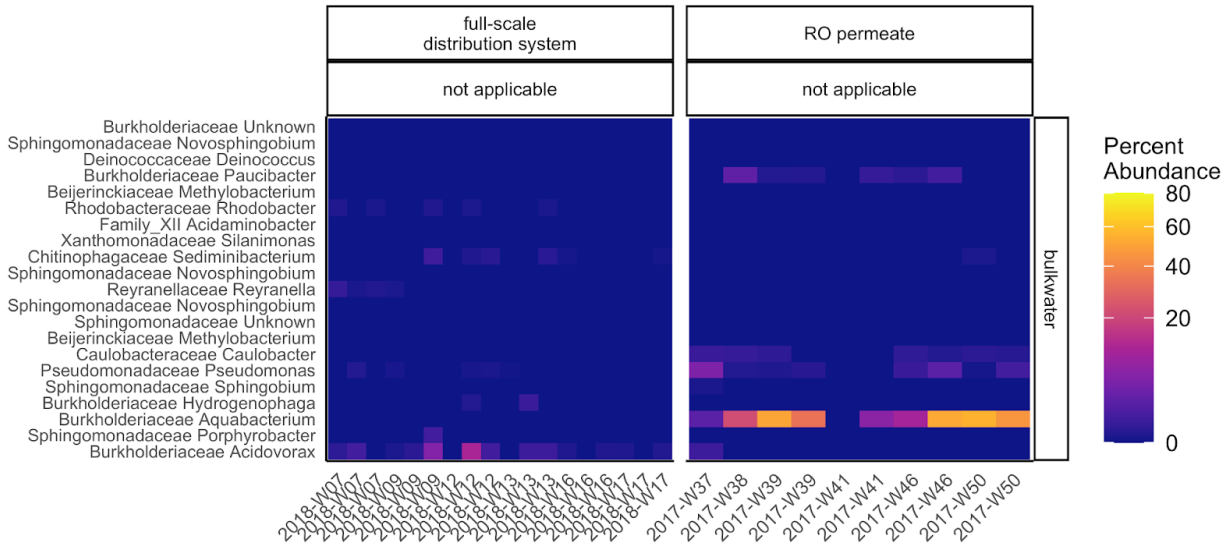

Figure S9: Heatmap of percent abundance (see scale on the right of the figure) of ASVs identified to significantly change in the transition loops in Phase 2 (rows) across sampling dates (columns - in the format yyyy-Wzz, yyyy is the year, and zz is a number from 01-52 that indicates which week of the year the sample was collected)

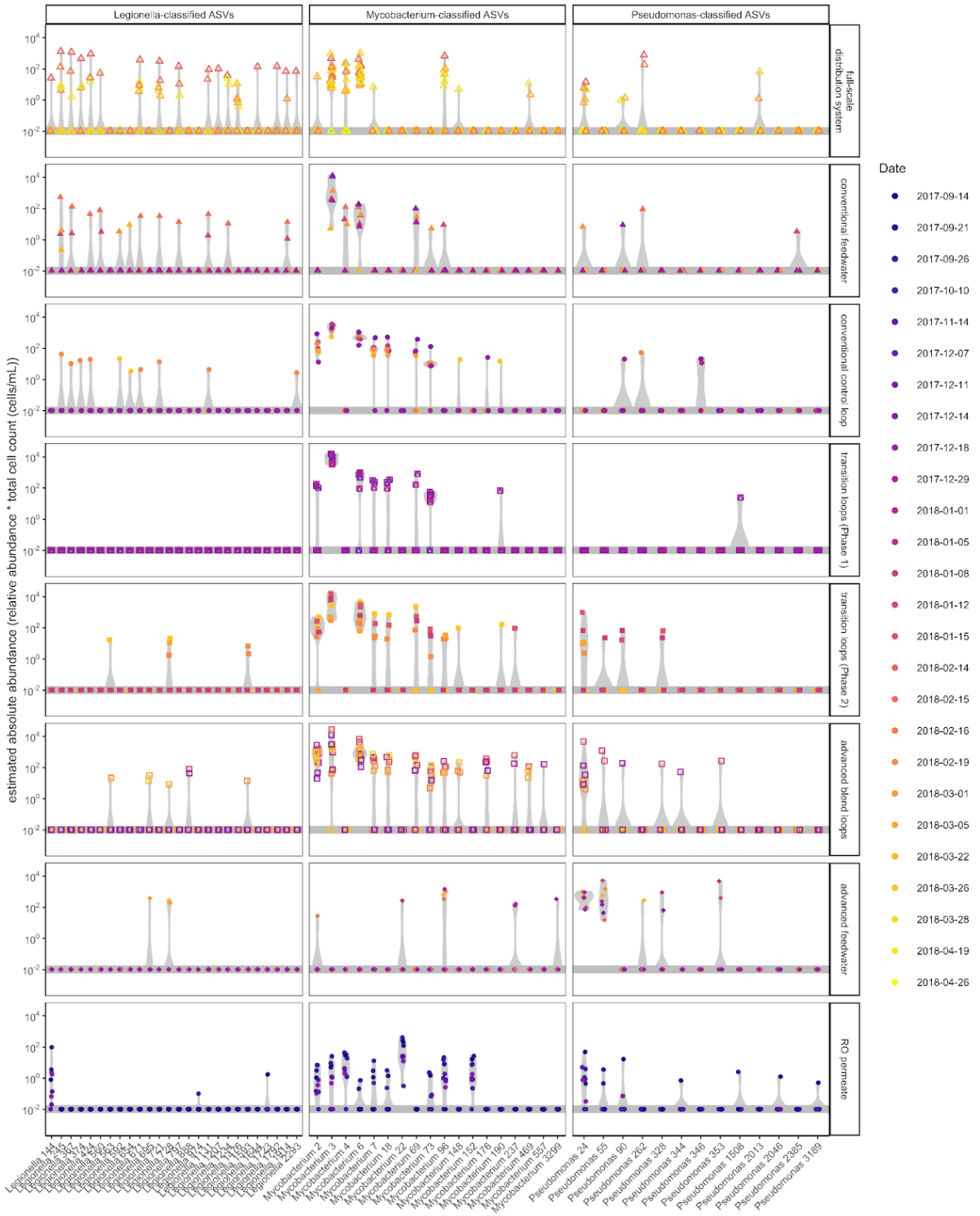

Figure S10: Estimated absolute abundances of potential opportunistic pathogens. ASVs (x-axis) are grouped by genera (column facets) and by sampling location (row facets). ASVs with 0% abundance are plotted as points on the gray horizontal line for visualization.

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