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Lateral Flow Sand Filters are Effective for Removal of Antibiotic Resistance Genes from Domestic Wastewater

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Abstract

The ability of lateral flow sand filters, used as on-site wastewater treatment systems (OWTS), to remove antibiotic resistance genes (ARGs), antibiotic resistant bacteria (ARB), and other relevant genetic markers (HF183, 16S rRNA, and *int1*) was assessed. Municipal wastewater was settled in a septic tank prior to loading into six pilot-scale lateral flow sand filters comprised of three different sand media types, at 5 and 30% slopes. The sand filters were sampled bi-weekly for: 9 ARGs and 3 other complimentary gene markers (*sul1*, *sul2*, *qnrS*, *tetO*, *ermB*, bla_{TEM}, $bla_{CTX\text{-}M}$, *mecA*, *vanA*, *int1*, HF183, 16S rRNA), and conventional microbial and water quality indicators, from July to November in 2017, and four times in the summer of 2018. The sand filters were observed to attenuate 7 of the ARGs to mostly below 2 log gene copies per mL. Log reductions ranging from 2.9 to 5.4 log were observed for the removal of absolute abundances of ARGs from septic tank effluent in 5 of the 6 sand filters. The fine-grained filter on the 5% slope did not perform as well for ARG attenuation due to hydraulic failure. The apportionment of cell-associated versus cell-free DNA was determined for the gene markers and this indicated that the genes were primarily carried intracellularly. Average log reductions of ARB with resistance to either sulfamethoxazole, erythromycin, or tetracycline were approximately 2.3 log CFU per mL within the filters compared to the septic tank effluent. This field study provides in-depth insights into the attenuation of ARB, ARGs, and their genetic compartmentalization in variably saturated sand OWTS. Overall, this type of OWTS was found to pose little risk of antimicrobial resistance contamination spread into surrounding environments when proper hydraulic function was maintained.

1. Introduction

Antibiotic resistance has become a leading threat to global public health as treatable pathogenic microbial infections have acquired resistance to conventional antibiotics (WHO, 2014).

Anthropogenic practices, including the use of clinical and agricultural antibiotics and antimicrobial product usage, can encourage the proliferation of antimicrobial resistance (AMR) by introduction of selective pressure on bacteria (Davies and Davies, 2010; Kolář et al., 2001). A hot spot for AMR development is in municipal wastewater treatment plants (WWTPs), where trace amounts of antibiotics taken within the general population are only partially metabolized, which leads to the development of AMR in bacterial communities within wastewater process streams (Munir et al., 2011). Antibiotic resistance in bacteria results from the expression of antibiotic resistance genes (ARGs), acquired as mobile genetic elements (MGEs) via horizontal gene transfer or as mutations via vertical transmission (Depardieu et al., 2007). Quantification of abundances of antibiotic resistant bacteria (ARB), ARGs and MGEs in WWTPs and receiving surface water environments have been conducted (Rizzo et al., 2013). ARG concentrations are typically reduced within many WWTPs; however, they persist in surface water systems downstream of effluent discharges (Freeman et al., 2018; McConnell et al., 2018a). Understanding the environmental dimension of AMR is important to enable the prediction of the spread of ARGs and AMR pathogens downstream of hot spots (Berendonk et al., 2015).

Removal, or conversely breakthrough, of ARGs and ARB within passive on-site wastewater treatment systems (OWTS) and variably saturated subsurface environments is less extensively studied. Despite this, antimicrobial products which encourage proliferation of AMR have been observed in septic tank effluent from OWTS (Conn et al., 2010). Unproperly treated wastewater in OWTS could pose a risk of bacterial contamination of surrounding drinking water resources (Crane and Moore, 1984). Approximately 15% and 20% of the population uses OWTS for provision of wastewater treatment in Canada and the United States, respectively. (Statistics Canada, 2015; EPA, 2018). OWTS are the second most frequent source of fecal contamination

of groundwater in the United States (Carroll et al., 2005). These can be a source of contamination for groundwater and adjacent surface water systems if they are not properly maintained. They may not be effective for attenuation of some types of contaminants of emerging concern such as pharmaceuticals and personal care products (Schaider et al., 2017). OWTS is often recommended to improve sanitation in developing nations due to relatively low cost, low maintenance requirements, and technical feasibility (WWAP, 2017). Contamination of groundwater with vectors of AMR from OWTS may be considered an issue of increased concern due to elevated reported susceptibility of developing regions to AMR (Ashbolt et al., 2013). While AMR prevalence in conventional centralized WWTPs is becoming increasingly better characterized; there remains a knowledge gap in the efficacy of low-tech treatment options to reduce risk of AMR contamination for developing countries (Bürgmann et al., 2018).

Treatment of ARGs with subsurface flow filter media has been studied by Anderson et al. (2015). The authors observed that ARGs and ARB associated with sulfonamide and tetracycline resistance adsorbed and persisted on the filter media, posing challenges for media disposal at the end of the filter life cycle (Anderson et al., 2015). Rural OWTS and municipal WWTPs were compared in China for ARG removal by Chen and Zhang (2013). The authors observed 1 to 3 log removal for ARGs in centralized WWTPs, but less effective removal for ARGs in rural OWTS; potentially due to lower overall abundances of ARGs in OWTS (Chen and Zhang, 2013). The removal performance of ARGs in a horizontal subsurface flow constructed wetland was studied by Nõlvak et al. (2013). ARG removal rates were higher in the wetland than observed in conventional WWTPs. ARG carrying microorganisms interacted with the wetland biofilm media; however, the exact attenuation mechanisms were not identified (Nõlvak et al., 2013).

89 The ARGs which encode for AMR may be present intracellularly, as cell-associated ARGs, or
90 extracellularly, as cell-free ARGs. Biologically active DNA may be transmitted, as it can be
91 transported in saturated soil environments with limited degradation, due to advective transport
92 and reduced efficacies of inhibitory DNA nucleases (Poté et al., 2003). Cell-free DNA
93 (extracellular DNA) can persist in soil environments for periods of up to several years
94 (Pietramellara et al., 2009). Characterization of cell-associated versus cell-free ARGs was
95 recently identified by Zhang et al. (2018) within a WWTP in China. Cell-associated ARGs were
96 observed to decrease and an cell-free ARGs increased as effluent progressed through the
97 treatment train suggesting that the cell-free ARGs may persist and spread potential AMR
98 contaminants in receiving environments. This is only a public health threat if the environmental
99 DNA is taken up and becomes integrated into the genome of viable bacterial hosts that are
100 pathogenic.

101 This study was undertaken to characterize the risk posed by OWTS in terms of introducing
102 contaminants of AMR into water resources. The objectives were to assess attenuation of ARGs
103 and ARB in lateral flow sand filters, which are an alternative to conventional septic fields, but
104 exemplify similar physical filtration and biological treatment mechanisms. Sub-objectives for
105 this study included an assessment of whether sand filter design factors (grain size and filter
106 slope) affect treatment performance. The apportionment of cell-associated versus cell-free ARGs
107 was quantified to assess whether the cell-free ARGs can penetrate through the filter more easily
108 than cell-associated ARGs. This study provides a comprehensive assessment of an array of
109 design configurations of OWTS for attenuation of AMR contamination, with a range of ARGs,
110 other complimentary gene markers, ARB, and assessment of the genetic compartmentalization of
111 ARGs.

2. Material and methods

2.1. Sand filters description

The experimental facility used in this study was located at the Bio-Environmental Engineering Centre (BEEC) in Truro, Nova Scotia, Canada. Six lateral flow sand filters (SFs) were installed at BEEC in 2004 and were constructed as per the *Nova Scotia Environment On-Site Sewage Disposal Technical Guidelines* (Nova Scotia Environment, 2013; Sinclair et al., 2013). The BEEC withdraws municipal wastewater from the Village of Bible Hill sewage collection line, which is then pumped into a septic tank multiple times daily. A pump is programmed to periodically dose the sand filters with septic tank effluent on a sub-daily basis via a flow splitter box and gravel distribution trench. The flow of effluent within the filters has been characterized as primarily tension saturated flow (Sinclair et al., 2013). Three different sand types were used in the construction of the filters, consisting of fine, medium, and coarse-grained sand; with saturated hydraulic conductivities of approximately 2.7×10^3 (SF1 and SF4), 6.3×10^3 (SF2 and SF5), and 1.2×10^4 cm/d (SF3 and SF6), respectively. Two slopes were assessed at 5 and 30%; design guidelines specify slopes ranging from 3 to 30% (Nova Scotia Environment, 2013). The grain size distributions are presented in the Supplemental Information (Figures S1 – S3). Each sand filter including the gravel distribution trench was fully lined on the sides and bottom with a high density polyethylene (HDPE) liner. The tops of the SFs were covered with filter fabric overlain by approximately 0.6 m of topsoil. The SFs were constructed at a 1:10 scale as per the dimensions illustrated in Figure 1. The effluent from each of the SFs was collected in a heated sampling building where each filter had a separate calibrated tipping bucket gauge for flow measurement. The influent was dosing rate was set by a programmable logic controller (PLC) to emulate a domestic household use with peaks in flow at 8 am and 7 pm (Figure S7 in the

Supplemental Information). The number of bucket tips were logged on a 30-minute frequency with a Campbell Scientific CR510 data logger (CSI, Logan, Utah, United States).

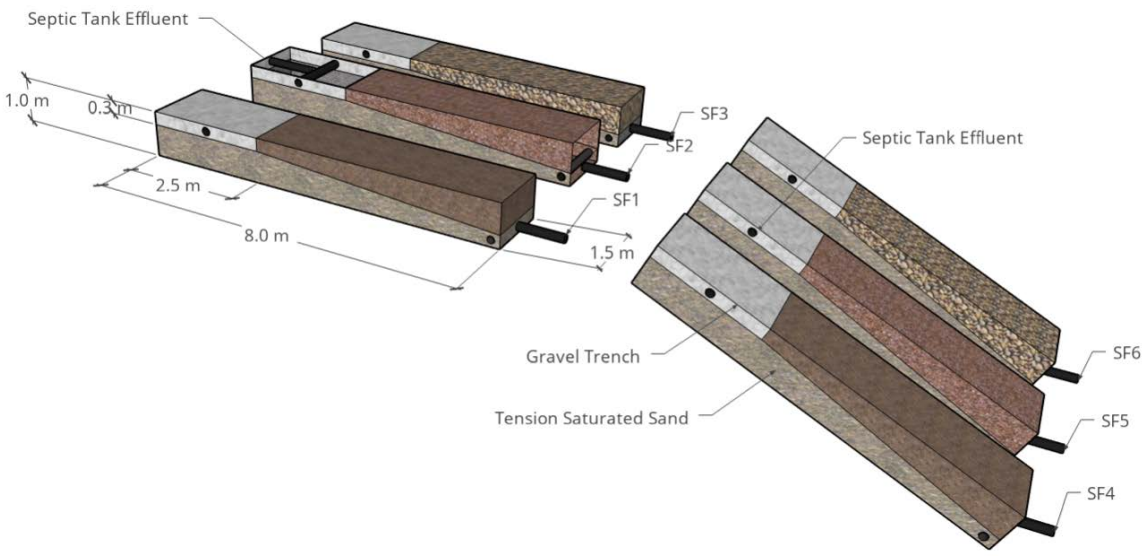


Figure 1. Schematic of the sand filter experimental layout (not to scale). Sand filter (SF)1 and SF4 filter media consist of fine-grained sand, SF2 and SF5 are medium grained sand, and SF3 and SF6 are coarse grained sand. SF1 – SF3 are on a 5% slope and SF4 – SF6 are on a 30% slope.

Average air temperatures near Truro were 18°C in July 2017 and ranged from a minimum of 12 to a maximum of 25°C; during November averaged 3°C, and ranged from -2 to 9°C. During July 2018, air temperatures near Truro averaged 21, and ranged from 14 to 27°C (Government of Canada, 2018).

2.2. Water sampling

All water samples were analyzed within 24 hours, except for antibiotics, which were analyzed within a one week holding time. Water samples for metals analysis were acidified with nitric acid to below pH 2 and store chilled for up to six months prior to analysis.

2.2.1. Conventional analysis

Water samples were collected from: the raw wastewater directly off the Bible Hill line as it discharged to a catch basin (1), the dosing box receiving effluent from the septic tank (1), and the filter effluent from each of the six (6) SFs. The hydraulic retention time (HRT) of the dosing box is approximately one day and the HRT of the septic tank is a minimum of two days. A total of eight (8) sample events were conducted on approximately a bi-weekly basis from July 5 to November 6, 2017, and analyzed for conventional wastewater parameters, as well as a suite of ARGs and associated AMR genetic markers. Four (4) additional sets of samples were collected during a two-day intensive sampling event that was conducted during a dry weather period on July 16 and July 23, 2018 to assess for daily-scale temporal variability. The ARG results were pooled for each day of this intensive sampling event for individual sample locations resulting in two (2) additional samples sets for a total of ten sample points (10). The intensive sample results were pooled due to low observed daily variability in concentrations as demonstrated in the results of the intensive sampling that are summarized in Table S3 of the Supplemental Information. During these two intensive sample event days additional microbial parameters including antibiotic resistant bacteria (ARB), and cell-associated and cell-free DNA were characterized. However, the ARB data collected during the intensive sampling event were not pooled.

Water samples were collected in sterilized 1L plastic sample bottles and transported in coolers on ice to the analytical laboratory at Dalhousie University in Halifax, Nova Scotia, Canada. General water quality indicators of temperature, dissolved oxygen (DO), specific conductance, and pH were made *in situ* for each sample collection event with a YSI600 handheld water quality sonde (YSI Inc., Yellow Springs, Ohio, United States). The sonde was calibrated as per manufacturer's

specifications. Conventional wastewater quality parameters that were analyzed for each sample included five-day carbonaceous biochemical oxygen demand ($CBOD_5$), total suspended solids (TSS), *Escherichia coli* (*E. coli*), total nitrogen (TN), total ammonia nitrogen (TAN), and total phosphorus (TP). These parameters were measured in accordance with standard methods (APHA, 2012). Total coliform and *E. coli* were enumerated with membrane filtration and Millipore mColiBlue24 broth® as per the standard instructions (Hach Company, Loveland, Colorado, United States). Quantification of a suite of 21 metals was conducted for all water samples with inductively coupled-mass spectrometry (ICP-MS) in accordance with APHA (2012).

2.2.2. Antibiotic analysis

The samples were analyzed for a suite of antibiotics once a month at Acadia University in Nova Scotia, Canada. These included: amoxicillin, cefaclor, cefprozil, cefdinir, levofloxacin, ciprofloxacin, azithromycin, clindamycin, clarithromycin, and triclocarban. See Supplemental Information for information on sample preparation and QAQC.

2.2.3. Genetic analysis

Approximately 25 mL of the raw wastewater and septic tank effluent (STE) water samples were filtered through a 0.45 µm pore size filter using a Millipore Vacuum Manifold and sterilized magnetic filtration funnels. Likewise, a measured volume of approximately 400 mL was filtered for the SF effluent. The DNA retained on the filters from the water samples was extracted with Qiagen DNeasy Powersoil Kits (Qiagen Inc., Toronto, Ontario, Canada). Following filtration, each filter was immediately placed in a Powerbead tube and subsequent processing steps were followed in accordance with manufacturer's specifications. Quantitative real-time polymerase chain reaction (qPCR) was used to enumerate the gene copy numbers of the following suite of

196 gene markers: class I integrase gene (*int1*), sulfonamide resistance genes (*sul1* and *sul2*),
197 methicillin resistance gene (*mecA*), vancomycin type A resistance gene (*vanA*), fluoroquinolone
198 resistance gene (*qnrS*), macrolide-lincosamide-streptogramin type B resistance gene (*ermB),*
199 tetracycline resistance gene (*tetO*), and class A β-lactamase genes (bla_{TEM} and $bla_{CTX\text{-}M}$). The
200 nine ARG markers were selected to represent the genes that confer resistance to the common
201 clinically prescribed antibiotics as identified by the Government of Canada (2016). The *int1* gene
202 was analyzed because it is commonly associated with MGEs and genes which confer resistance
203 to antibiotics (Gillings et al., 2015). The HF183 is a *Bacteroides* 16S ribosomal ribonucleic acid
204 (rRNA) gene marker that is human-specific and is used to measure human fecal pollution in
205 water environments (Seurinck et al., 2005); it was included in the gene scan to assess its utility as
206 an indicator marker of elevated presence of ARGs. The HF183 gene marker was assessed as per
207 the methodology described by McConnell et al. (2018a). The gene marker suite was quantified
208 using TaqMan qPCR on a Bio-Rad CFX96 Touch system (Bio-Rad, Herculer, California, United
209 States). The bacterial 16S rRNA gene copies were enumerated for each sample with SYBR
210 Green qPCR (Applied Biosystems Inc., Beverly, Massachusetts, United States). A
211 comprehensive description of the qPCR method development is found in Neudorf et al. (2017).
212 The primer and hydrolysis TaqMan probe sequences and cycling conditions are provided in the
213 Supplemental Information (Table S1). The limit of quantification (LOQ) and limit of detection
214 (LOD) of the gene markers are summarized in Table S2 in the Supplemental Information.

215 *2.2.4. Cell-associated and cell-free DNA analysis*

216 Cell-associated and cell-free DNA was enumerated for a small sub-set of the samples collected
217 in July 2018 according to a slightly modified version of a procedure introduced and described by

Zhang et al. (2018). Additional details on the methodology are provided in the Supplemental Information (Figure S5).

2.2.5. Antibiotic resistant bacteria enumeration

Total bacteria and antibiotic resistant bacteria in the raw wastewater, STE, and SFs samples from July 16 and July 23, 2018, were enumerated on agar plates containing no antibiotics (*i.e.*, total bacteria, control) and concentrations of either 50 mg/L sulfamethoxazole, 50 mg/L erythromycin, or 10 mg/L tetracycline (Mao et al., 2015). A spot plating method was used where three 20 µL drops (for a total volume of 60 µL) of serially diluted raw wastewater, STE, and SF effluent samples were placed on tryptone soy agar (TSA, Oxoid Ltd., Basingstoke, Hampshire, United Kingdom) plates, with or without each antibiotic at the defined concentrations, and incubated at 30°C for 24 hours. After incubation, the number of colonies were counted and recorded as log colony forming unit (CFU) per mL.

2.3. Sodium bromide tracer tests

Sodium bromide (NaBr) tracer tests were conducted on the SFs on July 30, 2018 during a dry weather period. These tests were conducted as per the methodology described in the Supplemental Information.

2.4. Statistical analysis

One-way analysis of variance (ANOVA) tests were performed on the absolute abundances of ARGs from the SF effluent over the study period to assess statistical difference at $p < 0.05$. A Shapiro-Wilk normality tested normality with the non-normality assigned at $p < 0.05$. The Brown-Forsythe method assessed for equal variance with significant differences in variances assigned at $p < 0.05$. When the assumption of normality was not met, a Kruskal-Wallis ANOVA on ranks was performed with significant difference between treatments assigned at $p < 0.05$. A

Tukey test was performed to assess significant differences between SF effluent absolute abundances and significance attributable at $p < 0.05$. The same statistical analysis was performed on the relative abundances of ARGs with addition of the raw wastewater and STE sample data. Throughout, $\bar{x}$ denotes mean of the sample. The potential for correlations between ARGs and other water quality indicators was of interest to assess whether there are water quality indicators associated with ARGs. To address this, a principal component analysis (PCA) was conducted on the 10-sample dataset with gene marker concentrations, conventional wastewater indicators, and metals concentrations in the raw wastewater, STE, and sand filter effluent. The metals that were excluded from the analysis included selenium (Se), silver (Ag), cadmium (Cd), antimony (Sb), cesium (Ce), and uranium (U), due to most measurements being below the detection limit (see Supplemental Information for metals data). The PCA data was log-transformed and analysed as a correlation matrix. The statistical analysis was performed with SigmaPlot version 13.0 statistical software (Systat software, Inc., San Jose, California, United States).

3. Results and discussion

3.1. Conventional parameters

The sand filters were effective at removal of the conventional wastewater parameters that were analyzed (Table 1). The average removal efficiencies for the filters ranged from 99 – 100% for $CBOD_5$, 91 – 100% for TSS, 5.2 – 6.7 log for *E. coli*, 27 – 37% for TN, and -1 – 60% for TP (negative value indicates net phosphorus production), which compared well with findings on this specific system by Wilson et al. (2011). Wilson et al. (2011) reported removal efficiencies of: 97 – 98% for $CBOD_5$, 82 – 97% for TSS, 4.3 – 5.2 log reduction for *E. coli*, 41 – 57% for TN, and 44 – 93% for TP. Wilson et al. (2011) attributed the primary removal mechanisms to physical filtration processes from the sand media and biological removal processes within the biological

264 zone (*i.e.*, biological mat) at the interface of the gravel distribution trench and the sand filter
265 media. The slight improvement in $CBOD_5$, TSS, and *E. coli* removal efficiencies may be
266 attributed to a matured biological zone over the past 7 years. Development of a biological mat is
267 characterized by a physical clogging of the pores in the distribution interface of a soil-adsorption
268 system; formation of this zone begins within the first few months of the operation of the soil-
269 adsorption system and gradually reaches an equilibrium (Beal et al., 2005).

Table 1. Summary of conventional wastewater parameter results presented as mean values ± standard deviation (n = 10).

Sample description	$CBOD_5$ (mg/L)	TSS (mg/L)	*E. coli*[a] (CFU/100mL)	TN (mg/L)	TAN (mg/L)	TP (mg/L)	Temp. (°C)	DO (mg/L)	pH
Raw	343 ± 138	295 ± 141	$3.4x10^6$ ± $3.5x10^6$	46 ± 23	45 ± 20	6.5 ± 2.9	15.9 ± 1.5	4.5 ± 2.7	7.4 ± 0.2
STE	219 ± 148	182 ± 128	$3.3x10^5$ ± $5.8x10^5$	54 ± 24	62 ± 26	9.6 ± 9.7	17.1 ± 1.2	2.8 ± 2.2	6.6 ± 0.1
SF1	2 ± 1	25 ± 20	1 ± 12	30 ± 6	0.1 ± 0.1	2.6 ± 3.2	16.5 ± 1.8	8.1 ± 1.2	6.1 ± 0.4
SF2	2 ± 1	5 ± 10	1 ± 0.6	30 ± 4	0.2 ± 0.3	3.3 ± 1.3	16.3 ± 1.8	10.3 ± 1.3	6.6 ± 0.2
SF3	2 ± 1	2 ± 2	1 ± 10	29 ± 6	0.1 ± 0.1	5.8 ± 5.0	16.2 ± 1.8	10.1 ± 1.2	6.4 ± 0.3
SF4	2 ± 1	1 ± 1	1 ± 2	33 ± 9	0.1 ± 0.1	1.7 ± 1.0	16.6 ± 1.7	9.9 ± 1.3	6.0 ± 0.2
SF5	2 ± 1	4 ± 3	4 ± 7	31 ± 5	0.1 ± 0.1	4.3 ± 3.5	16.0 ± 1.5	10.1 ± 1.1	6.0 ± 0.3
SF6	2 ± 1	3 ± 3	19 ± 339	29 ± 10	0.2 ± 0.5	6.6 ± 7.3	16.1 ± 1.7	9.9 ± 1.0	6.3 ± 1.2

[a]*E.coli* data is presented as geometric means.

3.2. Hydraulic characterization of filters

The HRTs of the sand filters are summarized in Table 2. SF1 had the longest HRT (~8 days), given that this filter had the lowest hydraulic conductivity as specified in Section 2.1, and was on a shallow slope; however, the deviation from the other filters warranted further examination. To investigate this, SF1 was partially excavated to the interface between the gravel trench and the sand media where the biological mat resided. SF1 was found to be partially clogged, with saturated conditions and ponded water within the biological mat (see Figure S4 in Supplemental Information). The finer grain size and low slope may have increased its vulnerability to failure. Saturated conditions in OWTS have been known to present a higher risk of conveyance of pathogens and ensuing human exposure (Beal et al., 2005). Average flows from each filter over the study period ranged from 108 to 152 L per day (See Figure S8 in Supplemental Information for the hydrographs over the study period).

Table 2. Summary of hydraulic characteristics of the sand filters determined from the bromide tracer tests.

Filter ID	Grain size	Slope (%)	HRT (days)	Mass recovery (%)	Time to peak (hrs)	Variance (dimensionless)
SF1	Fine	5	8	150[a]	154	0.16
SF2	Medium	5	4	73	36	0.79
SF3	Coarse	5	5	79	42	0.73
SF4	Fine	30	6	102	60	0.36
SF5	Medium	30	4	89	36	0.75

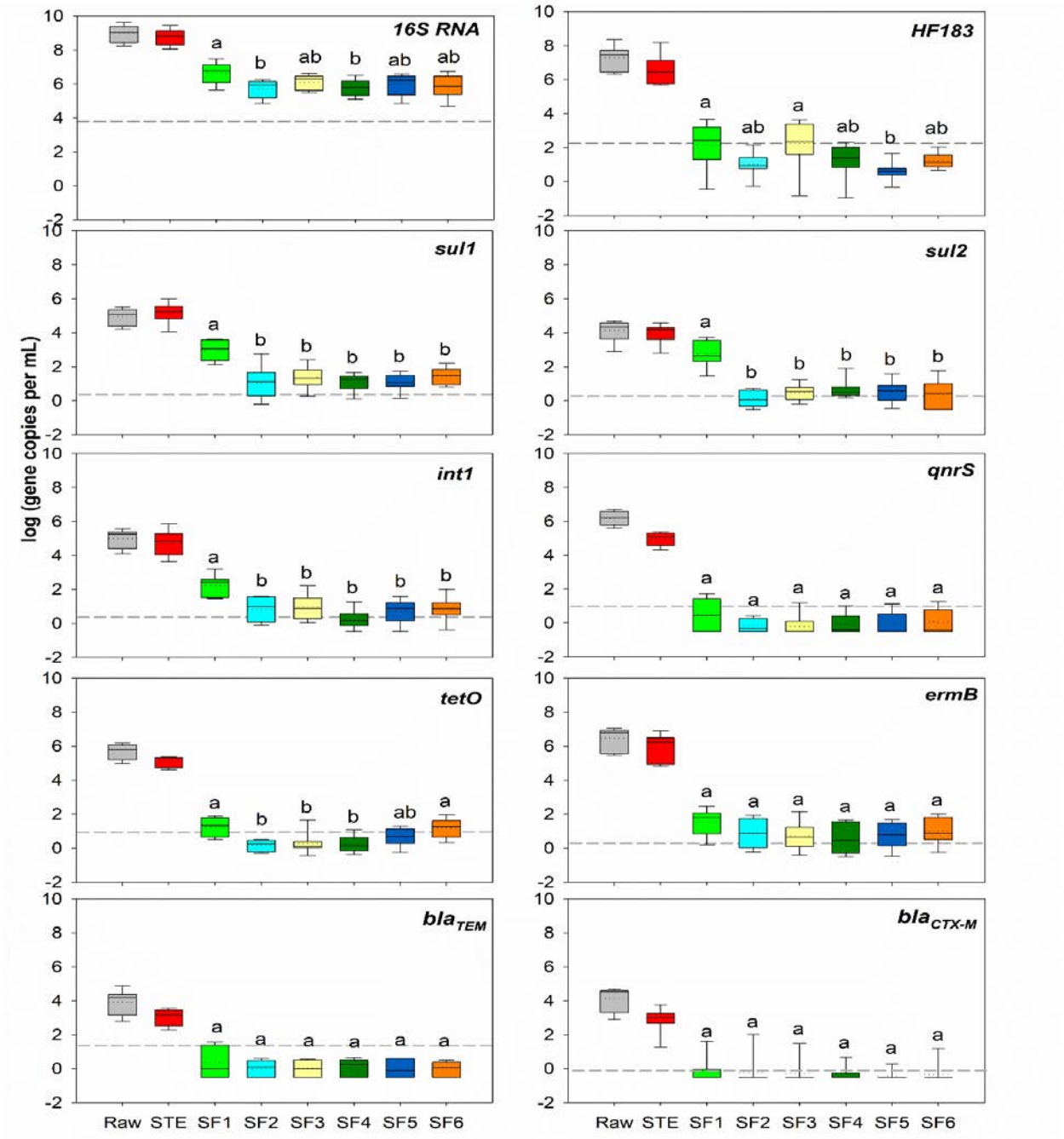
SF6	Coarse	30	3	86	30	0.84

[a]The mass recovery for SF1 was overestimated likely due to hydraulic failure of the filter and preferential flow in this filter (see Figure S6 in Supplemental Information).

3.3. Raw wastewater and septic tank effluent

3.3.1. Absolute and relative gene abundances

All the gene markers were present in the raw wastewater and STE, with absolute abundances well above the LOQs for all gene markers except for *vanA* and *mecA* (Figure 2). The vancomycin resistance gene, *vanA*, and in most samples the methicillin resistance gene, *mecA* were close to or below the LOQs. These two ARGs were not plotted in Figures 2 and 3, due to low levels (see Supplemental Information spreadsheet). The most abundant ARGs within the raw wastewater were *ermB* ($\bar{x}$ = 6.5 ± 0.7 log gene copies/mL), *qnrS* ($\bar{x}$ = 6.2 ± 0.4 log gene copies/mL), and *tetO* ($\bar{x}$ = 5.7 ± 0.4 log gene copies/mL). Overall, the septic tank removed minimal amounts of the gene markers from the effluent stream. Therefore, the most abundant ARGs within the STE were *ermB* ($\bar{x}$ = 5.9 ± 0.8 log gene copies/mL), and *sul1* ($\bar{x}$ = 5.2 ± 0.6 log gene copies/mL), *tetO* ($\bar{x}$ = 5.1 ± 0.3 log gene copies/mL). These ARG abundances in the STE were comparable in order of magnitude to raw and primary treated wastewater from other studies (Czekalski et al., 2012; McConnell et al., 2018b). In comparison to raw wastewater samples, there was no significant enrichment of ARGs in the STE (Figure 3). The highest relative abundances of ARGs in the STE were *ermB* ($\bar{x}$ = -2.5 ± -2.3 log gene copies for *ermB*/16S rRNA), and *sul1* ($\bar{x}$ = -3.4 ± -3.4 log gene copies for *sul1*/16S rRNA).



305

306 Figure 2. Absolute abundances of gene markers in the raw wastewater, septic tank effluent
307 (STE), and sand filter (SF) 1 – 6 for the duration of the study (n = 10). The middle lines represent
308 the median values, the dotted lines represent the means, the bottom and top of the boxes
309 represent the 25th and 75th percentiles, and the whiskers represent the 10th and 90th percentile of
310 the gene concentrations. The dashed line represents the limit of quantification for the sand filter
311 effluent. Difference in letters denotes significant difference of the gene absolute abundances at p
312 < 0.05 for the Tukey test. The raw wastewater and septic tank effluent samples were not
313 analyzed statistically as the differences in sand filter performance were of primary interest.

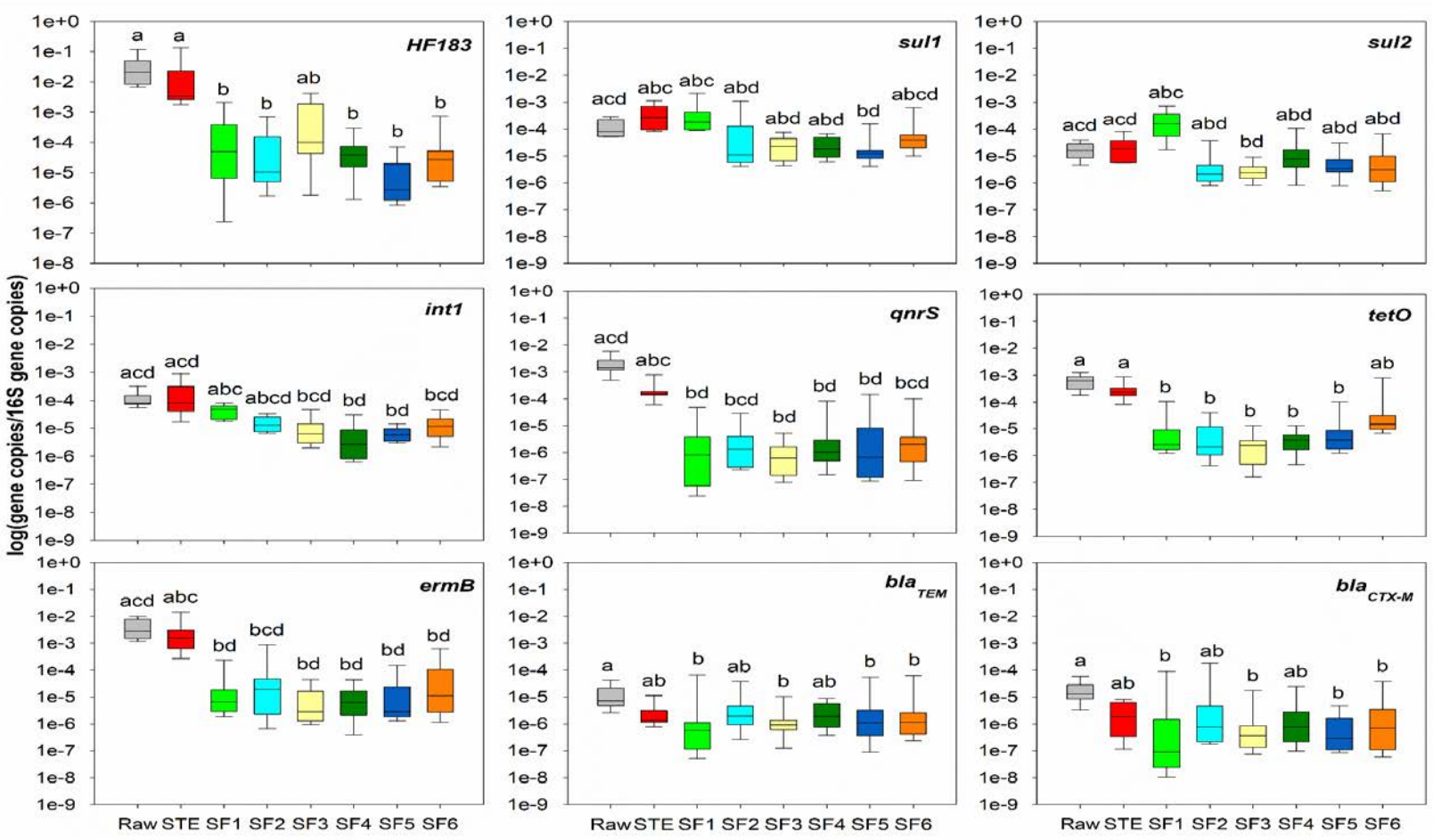


Figure 3. Relative abundances of gene markers in the raw wastewater, septic tank effluent (STE), and sand filters (SF) 1 – 6 for the duration of the study (n =10). The middle lines represent the median values, the dotted lines represent the means, the bottom and top of the boxes represent the 25th and 75th percentiles, and the whiskers represent the 10th and 90th percentile of the gene concentrations. Difference in letters denotes significant difference between the gene absolute abundances at $p < 0.05$ for the Tukey test.

3.4. Filter gene marker removal performance

3.4.1. Absolute and relative gene abundances

The sand filters performed effectively for the removal of ARGs from the STE as demonstrated with the absolute abundances illustrated in Figure 2. There were few significant differences between the filters apart from SF1, which removed significantly ($p < 0.05$) lower amounts of the ARGs. The effluent from all sand filters contained medians below LOQ levels for *qnrS*, *bla*$_{TEM}$, and *mecA*, and below LOD for *vanA*. No seasonal trends in ARG abundances were observed over the study period as shown in the Supplemental Information Table S5 in the spreadsheet.

Treatment of the STE in the SFs 2 – 6 resulted in the following average absolute removal of ARGs: 2.6 to 3.0 log removal for 16S rRNA, 4.3 to 6.0 log removal for *HF183*, 3.7 to 4.1 log removal for *sul1*, 3.3 to 3.8 log removal for *sul2*, 3.8 to 4.5 log removal for *int1*, 4.9 to 5.2 log removal for *qnrS*, 3.9 to 4.9 log removal for *tetO*, 4.9 to 5.4 log removal for *ermB*, 2.9 to 3.0 log removal for *bla*$_{TEM}$, and 3.1 to 3.3 log removal for *bla*$_{CTX\text{-}M}$.

Due to the decreased performance of SF1, it was considered separately from the aforementioned ranges with absolute removals of: $\bar{x}$ = 2.1 log removal for 16S rRNA, $\bar{x}$ = 4.4 log for *HF183*, $\bar{x}$ = 2.2 log removal for *sul1*, $\bar{x}$ = 1.2 log removal for *sul2*, $\bar{x}$ = 2.5 log removal for *int1*, $\bar{x}$ = 4.5 log removal for *qnrS*, $\bar{x}$ = 3.9 log removal for *tetO*, $\bar{x}$ = 4.4 log removal for *ermB*, $\bar{x}$ = 2.7 log removal for *bla*$_{TEM}$, and $\bar{x}$ = 3.0 log removal for *bla*$_{CTX\text{-}M}$. The lack of difference in the absolute abundances of the gene markers between the sand filters (except for SF1) suggested that the grain sizes in the three different sand mediums and two different slopes had little effect on the removal of the gene markers. It should be noted that the effective size (D_{10}) value of the three sand medias ranged from 0.12 – 0.18 mm. Therefore, the smaller particle sizes of the media were similar, which may have contributed to similar gene removal efficiencies.

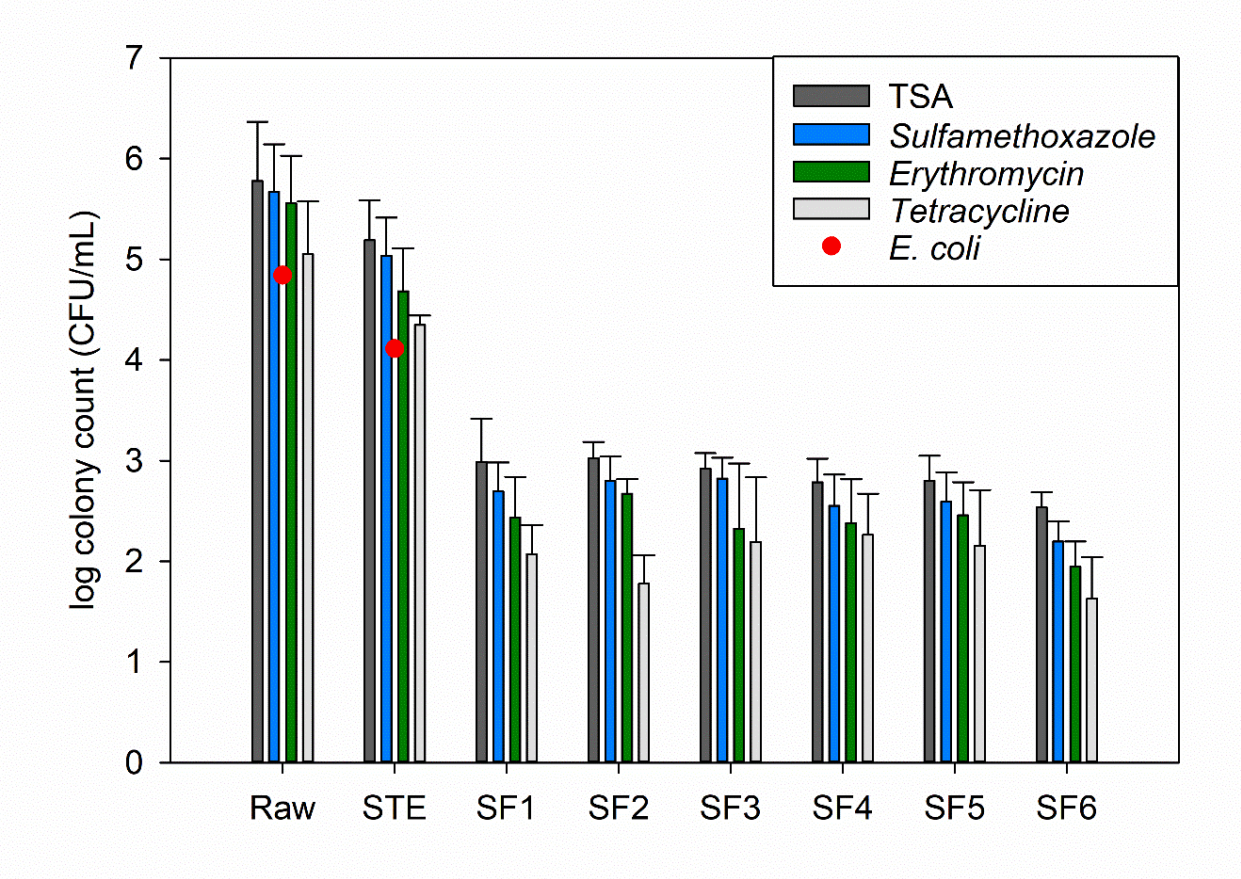
As noted above, the exception was SF1, which effected significantly ($p < 0.05$) less removal for *sul1*, *sul2*, and *int1* than all the other sand filters, resulting in levels that were well above the LOQ for these three ARGs. Likely, the decrease in attenuation of ARGs in SF1 was due to the suspected hydraulic failure of the filter. From an engineering perspective, this may suggest that an OWTS like SF1 with low hydraulic conductivity configurations on a shallow slope may present greater risk of failure and ARG breakthrough as they age.

The HF183 markers were generally below the LOQ except for SF1 and SF3 (Figure 2). This contrasts with the trends in some of the ARGs, for instance *sul1* and *ermB* have median absolute abundances consistently above the LOQs. Therefore, the utility of HF183 as an indicator for elevated ARGs associated with human fecal contamination may be useful, but not all encompassing.

In general, the relative abundances of the gene markers in filter effluent were significantly lower than in the raw wastewater and STE ($p < 0.05$; Figure 3). Some exceptions to this trend were evident, which included SF1 showing significant ($p < 0.05$) enrichments of *sul1*, *sul2*, and *int1* compared to the majority of the other sand filters. This enrichment of gene markers in SF1 is likely attributable to the hydraulic failure of this filter, which affected treatment performance. Overall, the relative abundances of the gene markers in the sand filter effluent represented a small percentage of the overall 16S rRNA gene abundances. These results suggest minimal gene marker enrichment when comparing the effluent samples, except for SF1 for *sul1*, *sul2*, and *int1*. Persistence of *sul* genes have also been reported in other types of wastewater treatment systems (McConnell et al., 2018b; Gao et al., 2012).

3.5. Antibiotic resistant bacteria

The treatment train was analyzed for ARB twice on July 16 and July 23, 2018, respectively (Figure 4). Bacteria that were resistant to antibiotics that were plated separately (*i.e.*, sulfamethoxazole, erythromycin, tetracycline) were present at comparable magnitudes ranging from 1.6 to 2.8 log CFU/mL in the SF effluent, down from levels of ~5 log CFU/mL in the raw wastewater. *E. coli* counts for the same sample events were low (< 1.2 log CFU/100mL) for the SF effluent, which indicated that the bacteria carrying the resistance to these antibiotics were likely different species than *E. coli*. *Sul* and *erm* which confer resistance to sulfamethoxazole and erythromycin were detected with absolute abundances above the LOQs in the sand filter effluent. This suggests that a portion of the *sul* and *erm* genes in the effluent would be associated with live bacteria. The qPCR analyses of a subset of the ARB colonies confirmed the presence of the relevant ARG markers (*sul1* and *sul2*, *ermB*, and *tetO*).



375

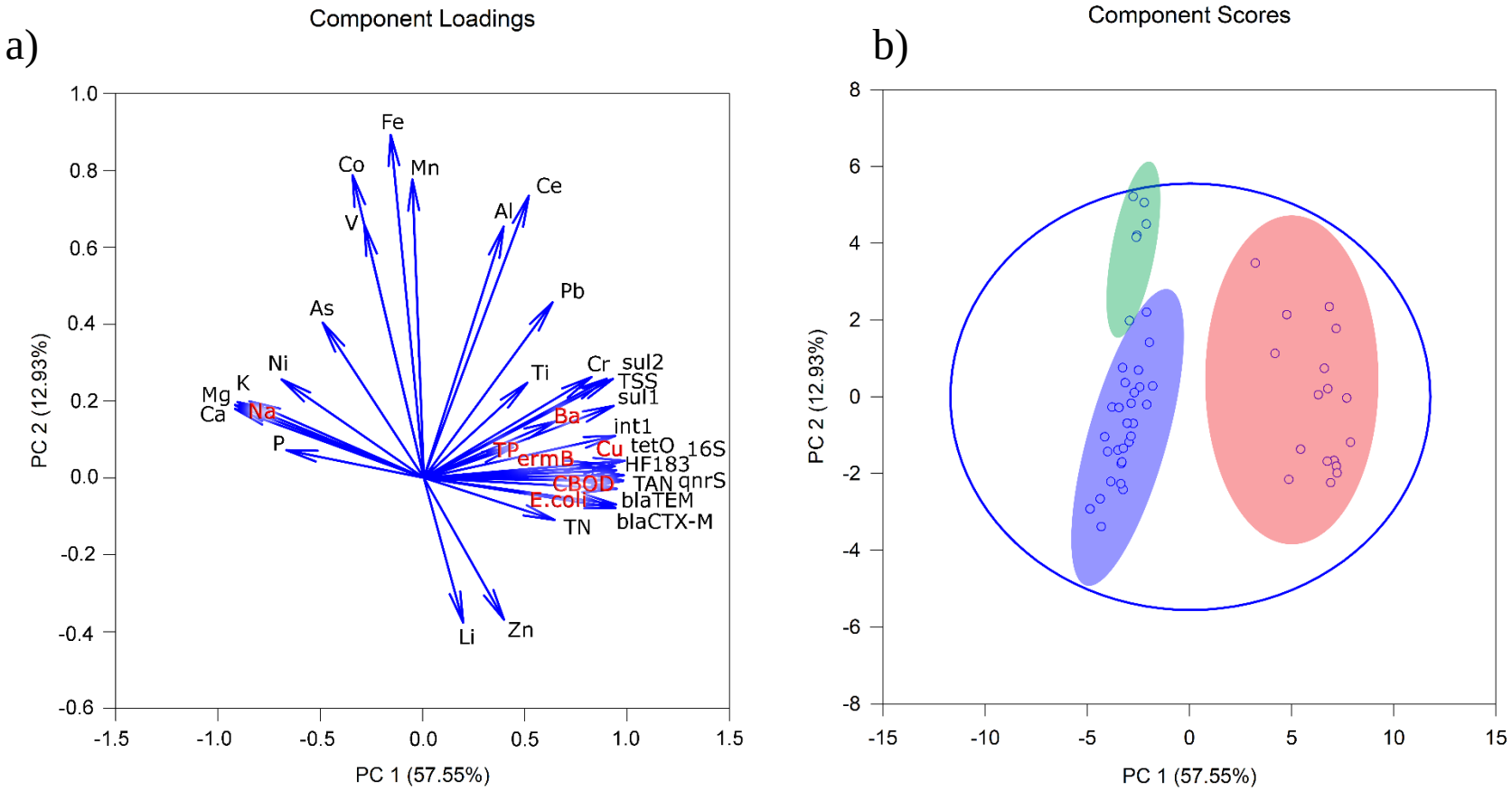
376 Figure 4. Geometric mean of total and antibiotic resistant bacteria in samples of raw wastewater,
377 septic tank effluent (STE), and sand filter (SF) effluents (sampled on July 16 and 23, 2018, n =
378 4). The error bars represent one standard deviation. The *E. coli* is presented as the geometric
379 mean of two samples collected on July 16 and 23, 2018. The *E. coli* concentrations for the SF
380 effluent were all below 1 CFU/mL.

381 *3.6. Correlations between gene markers and water quality parameters*

382 There was a positive correlation between the gene markers and the conventional wastewater
383 quality indicators (Figure 5a). *E. coli* showed a positive correlation to the gene markers, which
384 is anticipated as bacteria such as *E. coli* can house selected gene markers intracellularly. Several
385 heavy metals were positively correlated with the gene markers, which included chromium (Cr),
386 barium (Ba), and copper (Cu), which may indicate co-selection for resistance to metals and
387 ARGs in bacteria. Chromium and copper were elevated in the raw wastewater and STE which
388 may have been an artifact of metals originating from household plumbing fixtures. Co-selection

389 of ARGs and heavy metal resistance genes in municipal wastewater have been observed by Di
390 Cesare et al. (2016), and specifically co-selection of tetracycline and copper was observed by
391 Amachawadi et al (2013). Sodium (Na), magnesium (Mg), calcium (Ca) and potassium (K) were
392 observed to be negatively correlated with the gene markers and conventional wastewater quality
393 indicators. This inverse relationship may be explained by relatively lower concentrations
394 observed for these cations in the raw wastewater and STE, and an elevated concentration in the
395 sand filter effluent. Calcium carbonate ($CaCO_3$) dissolution is characteristic of septic field
396 environments as a buffer for NH_4^+ oxidation, which results in increased Ca^{2+} concentrations in
397 the effluent, and other major cations may also exhibit similar mineral dissolution, or cation
398 exchange reactions (Wilhelm et al., 1994). The scores plot in Figure 5b shows the overall
399 difference in concentrations between the raw wastewater and STE samples; which were
400 generally grouped together, and the sand filter samples which were clustered together, except for
401 SF1. This confirms the degree of system characterization (n = 10 sample events) was adequate to
402 capture variability in water quality.

403



404 Figure 5. Principal component analysis (PCA) of the gene marker concentrations and water quality indicators along the treatment
405 train. This illustrates the: a) loadings plot of the gene markers and other parameters (n = 10 sample events), use of red text is for
406 contrast; and b) scores plot of the PCA results of the wastewater sampling. Ellipses denote groupings of scores of sand filters 2 – 6
407 (blue); sand filter 1 (green); and the raw wastewater and septic tank effluent samples (red). The numbers in brackets represent the
408 percentage of variance described in the dataset by the first and second components.

3.7. Apportionment of cell-associated and cell-free gene markers

The enumeration of total, cell-associated, and cell-free DNA for each gene marker within the treatment train are presented in Table 3. Cell-associated DNA represented the greatest apportionment of DNA for all the gene markers and throughout the treatment train. All cell-free DNA observed in the analysis were either below LOQ or LODs. The raw wastewater and STE had negligible apportionment of cell-free DNA, and the sand filters contained cell-free DNA levels below the LODs, including the poorly performing SF1. This indicates that the gene markers that were measured throughout the treatment train resided primarily inside bacterial cells (*i.e.*, intracellularly). This finding indicates that this type of treatment system is at low risk of spreading cell-free ARGs.

Table 3. Summary of average gene marker concentrations in the total, cell associated, and cell-free DNA fractions for raw wastewater, septic tank effluent (STE) and sand filters (SF) 1 – 3 samples collected on July 16 and July 23, 2018. Bolded numbers indicate absolute abundances above the LOQ, italicized numbers are below the LOQ, <DL means are below the detection limit .

	Sample ID														
	Raw			STE			SF1			SF2			SF3		
Gene marker	Total	Cell-associated	Cell-free	Total	Cell-associated	Cell-free	Total	Cell-associated	Cell-free	Total	Cell-associated	Cell-free	Total	Cell-associated	Cell-free
	Log gene copies per mL														
16S rRNA	**9.5**	**8.5**	*1.4*	**9.2**	**8.0**	*1.1*	**6.8**	**6.4**	<DL	**6.3**	**5.3**	*0.8*	**6.4**	**5.7**	<DL
HF183	**6.7**	**6.4**	*0.6*	**5.8**	**5.6**	*0.5*	*2.1*	<DL	<DL	**3.0**	**2.3**	<DL	**2.6**	*1.1*	<DL
sul1	**5.1**	**4.3**	*1.1*	**4.5**	**4.0**	*1.2*	**3.0**	**2.4**	<DL	**1.3**	<DL	<DL	**1.9**	<DL	<DL
sul2	**4.5**	**3.8**	<DL	**4.1**	**2.8**	*1.0*	**3.0**	**2.4**	<DL	<DL	<DL	<DL	**1.2**	<DL	<DL
int1	**5.6**	**4.5**	*1.1*	**4.1**	**3.6**	<DL	**2.7**	**1.7**	<DL	<DL	<DL	<DL	**1.5**	<DL	<DL
qnrS	**6.1**	**5.7**	<DL	**5.0**	**4.2**	<DL	**3.6**	<DL	<DL	**1.7**	<DL	<DL	**2.5**	<DL	<DL
tetO	**5.4**	**5.0**	<DL	**5.0**	**4.6**	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL
ermB	**6.1**	**5.6**	<DL	**5.2**	**4.9**	<DL	**1.3**	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL
bla_{TEM}	**3.3**	**2.9**	<DL	*2.4*	*2.1*	<DL	*1.1*	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL
bla_{CTX-M}	**4.0**	**3.0**	<DL	**2.6**	**2.0**	<DL	**1.2**	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL
mecA	*1.7*	*1.0*	<DL	*1.5*	<DL	<DL	**1.0**	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL
vanA	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL	<DL

3.8. Antibiotics

The antibiotic data is in Table S4 of the Supplemental Information. All the antibiotics were detected at least once in the treatment train during the study period. However, during many sample events, several of the antibiotics were not detected. An exception was clindamycin, which was often present in detectable concentrations within the treatment train. In clinical settings, the *erm* gene can confer resistance to clindamycin as well (Levin et al., 2005). Figure 2 shows that *ermB* was often present above LOQ in the effluent of all the sand filters. Intermittingly, all the antibiotics except for azithromycin, were detected in the sand filter effluents. The chemical stability of antibiotics varies, and some are quick to degrade, which may explain absence in the effluent. There was no direct relationship between antibiotics and ARGs because of the ephemeral nature of the presence of the antibiotics in the influent. The bacteria within the septic tank and biological mat acquire resistance through repeated intermittent exposure over time.

4. Conclusions

This study demonstrated lateral flow sand filters help to reduce the risk of AMR contamination from OWTS when the hydraulics are properly functioning. Most of the ARGs assessed were removed to below 2 log gene copies per mL for absolute abundance. Grain size of the filtration media or filter slope had no observable impact on the efficacy of the removal of ARGs except for SF1. The exception of SF1 was due to partial hydraulic failure of the system as evidenced by clogging and water retention on the biological mat. In SF1, significantly ($p < 0.05$) less removal of *sul1*, *sul2* and *int1* were observed in comparison to the other sand filters and therefore elevated ARGs passed through into the filter effluent. This highlights the need for inspection and maintenance of these types of OWTS as they age.

ARGs were mostly found to be present intracellularly in the bacteria as opposed to extracellularly. This type of OWTS system poses low risk of cell-free DNA breakthrough and subsurface transport. ARB, resistant to either sulfamethoxazole, erythromycin, or tetracycline, were observed to undergo an average of 2.3 log reduction across the sand filters. Of importance, the ARB were present in the sand filter effluent with counts ranging from 1.6 to 2.8 log CFU per mL. Concurrently, these samples generally contained non-detectable levels of *E. coli*. Therefore, sole reliance on *E. coli* as an indicator may be inadequate to capture the risk of releasing AMR pathogens from mal-functioning OWTS.

Future research would be useful to characterize the filter biological mat, specifically examining ARGs and microbial community structure using metagenomics. This would enable further understanding and potential optimization of the biological mat attenuation mechanisms in filtration technology development. Understanding of fate of ARGs in saturated environments would also be useful for further characterization of risk to groundwater resources.

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