# Evaluating Antibiotic Resistance Gene Correlations with Antibiotic Exposure Conditions in Anaerobic Membrane Bioreactors

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# Abstract

Anaerobic membrane bioreactors (AnMBRs) are an emerging technology with potential to improve energy efficiency and effluent reuse of mainstream wastewater treatment. However, their contribution to the proliferation of contaminants of emerging concern, such as antibiotic resistance genes (ARGs), remains largely unknown. The purpose of this study was to determine the effect of select influent antibiotics at varying concentrations on the presence and abundance of ARGs in an AnMBR system and its effluent. Quantification of targeted ARGs revealed distinct profiles in biomass and effluent, with genes conferring resistance to different antibiotic classes dominating in biomass (macrolides) and effluent (sulfonamides). Effluent *sul1* gene abundance was strongly correlated with abundance of *intI1*, signifying the potential importance of mobile genetic elements in ARG release from AnMBR systems. The addition of specific antibiotics also affected normalized abundances of their related ARGs, exemplifying the potential impact of selective pressures at both low (10 µg/L) and high (250 µg/L) influent antibiotic concentrations.


# 1. Introduction

Despite the longstanding benefits of antibiotic use for the treatment of infectious diseases, their unwanted persistence in wastewater has intensified antibiotic resistance in natural microbial communities.[1-3] Approximately 23,000 deaths in the US and 25,000 in Europe have been attributed to antibiotic resistant bacteria (ARB), along with approximately $1 billion and $1.5 billion in annual healthcare costs, respectively.[4, 5] Reports have linked antibiotic loading from hospitals and farms to increases in resistance within downstream surface water, soil, and groundwater.[6] Water reuse (a more common practice given persistent water scarcity) elevates such threats by reducing buffers between wastewater and potential human exposure.[7] These circumstances have created a sense of urgency worldwide, with antibiotic resistance cited as one of the most critical human health risks.[4] Moreover, sensitive (nonresistant) bacteria can acquire resistance mechanisms from ARB via the horizontal exchange of mobile genetic elements (MGEs) containing antibiotic resistance genes (ARGs; e.g., plasmids, integrons, and transposons).[4]

Wastewater treatment systems (both municipal and agricultural) are the primary gateway for antibiotic release to the environment.[6] The high density of microorganisms in these systems promotes antibiotic resistance proliferation via vertical and horizontal gene transfer when exposed to sub-lethal levels of antibiotics.[8, 9] Studies have demonstrated that higher solids retention times (SRTs) increase ARG abundance in activated sludge of both membrane-based and conventional treatment processes.[10-12] Further, studies have evaluated various antibiotic types relative to abundance of their associated ARG, with some showing positive correlations and others showing negative correlations or none at all.[13-15]

Anaerobic treatment remains significantly understudied with respect to antibiotic resistance.[16] Anaerobic membrane bioreactors (AnMBRs) are emerging as the forefront anaerobic technology for mainstream treatment, promoting both energy recovery and agricultural effluent reuse while achieving similar treatment performance to aerobic processes at a range of operational temperatures.[17, 18] Theoretically, AnMBRs can lessen the spread of antibiotic resistance by minimizing excess sludge production due to the significantly lower yields of anaerobic microorganisms relative to their aerobic counterparts in activated sludge. Although this inherently results in extended SRTs (shown to increase ARG abundance in aerobic biomass), longer SRTs in anaerobic systems may reduce ARG presence.[19, 20]

Few studies have evaluated AnMBRs for their ARB and ARG reduction capacity. Notably, recent work by Kappell et al. exhibited log removal values of greater than 3.5 for *sul1*, *ermB*, and *tetO* during AnMBR treatment of primary clarifier effluent.[21] Further, recent work by Cheng et al. showed that both ARB and ARG removal was significantly improved by subcritical membrane fouling in an AnMBR, implying that membrane biofilms may play an integral role in their reduction.[22] However, no studies to date have systematically considered the presence and concentration of specific antibiotics on ARG or ARB abundances in AnMBR biomass and effluent, despite their observed correlations in other wastewater systems. Further, antibiotic concentration is of particular relevance for evaluating system applicability to different wastewater source types (e.g., domestic vs. hospital wastewater). In the present study, ARGs and ARB were quantified in an AnMBR system across varying influent antibiotic concentrations for three different antibiotic types.

# 2. Materials and methods

## 2.1. Bench-scale anaerobic membrane bioreactor operation

A bench-scale AnMBR consisting of a 5 L working-volume continuously stirred tank reactor (CSTR) (Chemglass Life Science, Vineland, NJ) was operated at 25 °C. Three separate membrane housings were submerged in the reactor, each including a flat-sheet silicon carbide (ceramic) microfiltration membrane (Cembrane, Denmark) with 0.1 µm pore size. The effective membrane area of each module was approximately 0.015 $m^2$. The AnMBR was inoculated with sludge from a mesophilic anaerobic digester at the Joint Water Pollution Control Plant (Carson, CA). AnMBR influent was a synthetic wastewater (Table S1) representative of domestic wastewater in the US.[23] The use of synthetic wastewater in this study allowed for the direct control and observation of specific antibiotic addition without the incidental influence of background antibiotic, ARB, or ARG occurrence.

After steady AnMBR performance was reached, defined as consistently low effluent COD (<40 mg/L), stable biogas production, and high methane content (>60%) over at least 10 days of operation, three antibiotics including sulfamethoxazole (sulfonamide), erythromycin (macrolide), and ampicillin (β-lactam), were added to the influent in independent sequential phases at incremental concentrations of 10, 50, and 250 µg/L (10 days at each concentration) to represent typical antibiotic level ranges in domestic and hospital wastewater discharges.[24, 25] Before each antibiotic phase, membrane modules were removed for physical and chemical cleaning using 0.5% (v/v) NaOCl solution to prevent membrane fouling from influencing observations between phases (details provided in SI). After membrane cleaning, the AnMBR was operated for one week prior to commencement of the next antibiotic phase. Details regarding AnMBR operation and performance analysis methods are provided in the Supporting Information (SI).

## 2.2. Antibiotic Quantification

All antibiotics were obtained from Sigma-Aldrich (>99% purity). Quantification of antibiotics was achieved using matrix-matched external calibration to correct for any suppression or enhancement effects of the influent and effluent sample matrices. Sample antibiotic concentrations were analyzed by direct injection liquid chromatography mass spectrometry with electrospray ionization (LC-ESI-MS) on a 6560 Ion Mobility Quadrupole Time-of-Flight (IM-QTOF) LC-MS system (Agilent) using 1290 Infinity UHPLC, Dual Agilent Jet Stream (ASJ) ESI, and EclipsePlus C18 column (2.1mm; 50 mm; 1.8um). Method detection limits (MDLs) and practical quantitation limits (PQL) for each targeted compound were estimated using minimum signal to noise ratios of 3:1 and 10:1, respectively. PQLs were <0.1 µg/L for all three antibiotics based on

compound-specific optimization of LC-ESI-MS conditions. Details of sample preparation procedures, optimized LC program, and MS operational conditions are presented in the SI.

### 2.3. ARG quantification by qPCR

AnMBR biomass and effluent were both sampled biweekly for DNA extraction. For biomass samples, 2 mL of suspended sludge was centrifuged at 5,000g for 10 minutes, supernatant decanted, and stored at -80 °C. A freeze dry system (FreeZone 2.5 Liter Freeze Dryer, Labconco, Kansas City, MO) was used to lyophilize 50 mL effluent samples, which were then stored at -80 °C prior to DNA extraction. DNA extraction was performed using a Maxwell 16 Blood DNA Purification kit (Promega, Madison, WI) according to manufacturer specifications. Extracted DNA concentration and quality were evaluated spectrophotometrically using a BioSpectrometer (Eppendorf, Hamburg, Germany).

Quantitative polymerase chain reaction (qPCR) was performed on a LightCycler 96 (Roche, Basel, Switzerland) targeting a suite of 9 ARGs spanning a range of antibiotic classes based on the most common ARGs observed in domestic wastewater in previous studies.[19, 26, 27] Targeted ARGs included genes conferring resistance to sulfonamides (*sul1* and *sul2*), macrolides (*ermF* and *ermB*), β-lactams (*oxa-1*, *ampC*, and *mecA*), and tetracycline (*tetW* and *tetO*), as well as *intl1* which encodes for class 1 integrons. The *rpoB* gene was also quantified for ARG normalization due to its ubiquity as a single copy gene, preventing potential biases with multiple 16S rRNA operon copy numbers. qPCR reactions were carried out in 20 µL reactions containing 10 µL qPCR master mix (Forget-Me-Not EvaGreen, Biotium, Fermont, CA), forward and reverse primers at a final concentration of 0.25 µM each, 1 µL of DNA template and $ddH_2O$. Each reaction was performed in triplicate. Thermal cycling was varied for each ARG targeted, with details provided in the SI.

### 2.4. ARB quantification

Total bacteria and ARB in the effluent were enumerated using the heterotrophic plate count (HPC) method.[28] Nutrient agar was used for all HPC plating. Ampicillin, tetracycline, erythromycin, and sulfamethoxazole were added to the nutrient agar at 16, 16, 50.4, and 18.1 µg/mL, respectively, based on previously used ARB quantification methods.[29, 30] Effluent samples from the permeate of all three membrane units were collected in a sterile microcentrifuge tube and diluted with 1X PBS to the expected range necessary to achieve plate counts in the range of 30-300 colony forming units (CFUs) per plate. All diluted effluent samples were then plated in duplicate. Plates were incubated at 35 °C for 48 hours prior to CFU enumeration.

### 2.5. Statistical analysis methods

To determine significance of changes in ARG abundance at the different influent antibiotic concentrations, a 2-tailed unpaired student's t-test was carried out between all phase-adjacent data sets. To evaluate for significant linear correlation between data, Pearson correlation was employed using MAXSTAT Lite 3.6 over a 95% confidence interval. Correlation analysis was performed between all ARGs, ARB, and antibiotic concentrations over the entire operational period. Strong and weak correlations were identified based on the Pearson coefficient (ρ) as $\rho > 0.7$ or $\rho < -0.7$ for strong correlation and $0.3 < \rho < 0.7$ or $-0.7 < \rho < -0.3$ for weak correlation. The analysis was carried out for biomass and effluent separately.

# 3. Results and discussion

## 3.1. AnMBR performance and antibiotic removal was robust throughout operation

Chemical oxygen demand (COD) removal in the AnMBR averaged 93±3.1% throughout operation resulting in an effluent COD of 35±17 mg/L. Mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) concentrations were relatively constant at 8.2±0.5 g/L and 6.6±0.5 g/L, respectively. Average biogas production was 726±39 mL/d with an average methane content of 76.4±5.7%. Membrane performance was consistent throughout the experiment with an effective transmembrane flux of 7 L/m$^2$/h and transmembrane pressure of <25 kPa. Sulfamethoxazole and ampicillin addition to the influent of the AnMBR both coincided with steady increases in biogas methane content ($p$<0.036; Figure 1A). Although it was unclear whether this trend was a result of antibiotic addition, previous work has shown that methanogens can degrade sulfonamide antibiotics as a co-substrate.[31] However, sulfamethoxazole COD at the concentrations used in the present study was negligible relative to influent COD. The impact of antibiotics on methanogen activity deserves further research. Antibiotic removal rates were high for all three of the antibiotics tested (> 67%; Figure 1B). Removal rates for sulfamethoxazole and erythromycin were in the range of 71-85% and 67-88%, respectively, with no significant trends. Ampicillin removal rates were significantly higher, starting above 94% upon initial addition and increasing to over 98%. These relatively high parent compound removal rates are in general accordance with previously reported ranges for anaerobic treatment,[32-35] which in the cases of sulfamethoxazole and ampicillin, signify an advantage over removal efficiencies of conventional activated sludge-based systems.[36, 37]

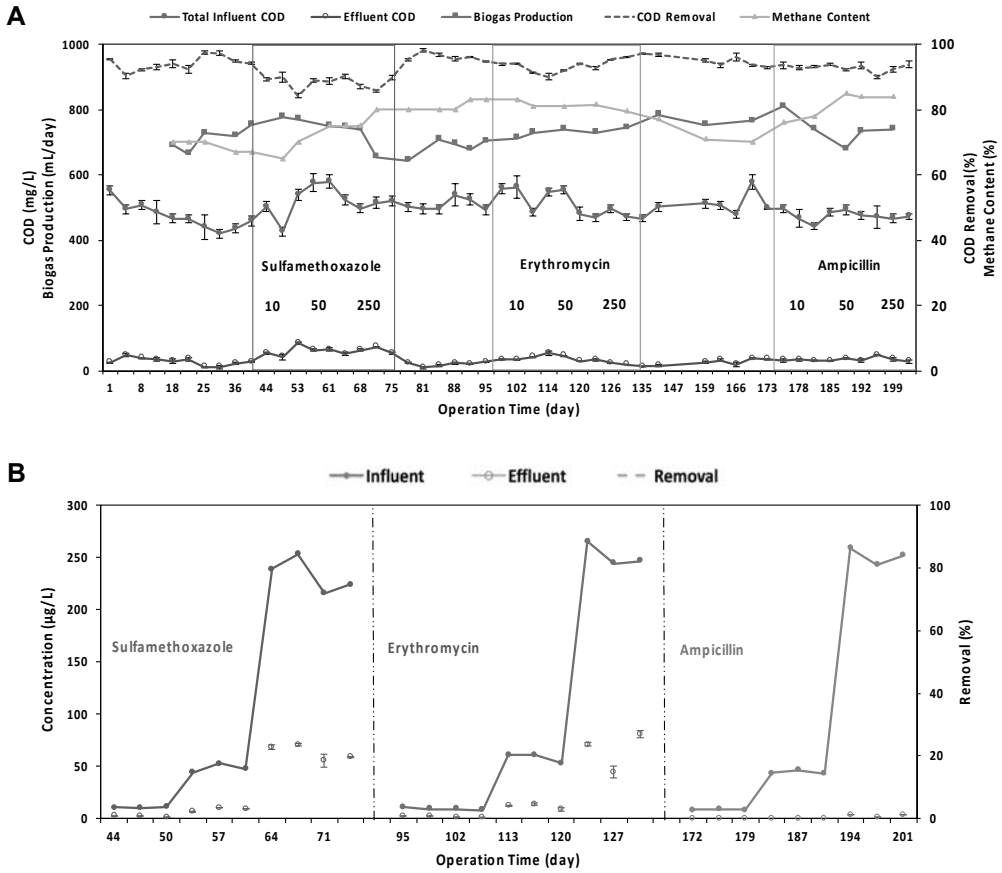


**Figure 1.** (A) Performance of AnMBR in COD removal and biogas production during the addition of sulfamethoxazole, erythromycin and ampicillin at increasing concentrations; (B) Fate of sulfamethoxazole, erythromycin and ampicillin in AnMBR. Error bars represent the standard deviation of the results obtained from replicate samples

### 3.2. AnMBR effluent exhibited a unique ARG profile compared to the biomass and membrane biofilm

The ARG profile of the biomass, biofilm, and effluent revealed significant differences independent of the specific antibiotic addition phases. Exemplifying this, a comparison of biomass and effluent samples at 250 µg/L sulfamethoxazole in the influent, along with the corresponding membrane biofilm (taken directly after 250 µg/L phase), revealed that biomass and biofilm ARG profiles were similar to each other and both remarkably different from the effluent (Figure 2). Analysis of subsequent antibiotic phases (erythromycin and ampicillin) revealed a similar relative distribution of ARG profiles across sample type (Figure S1).

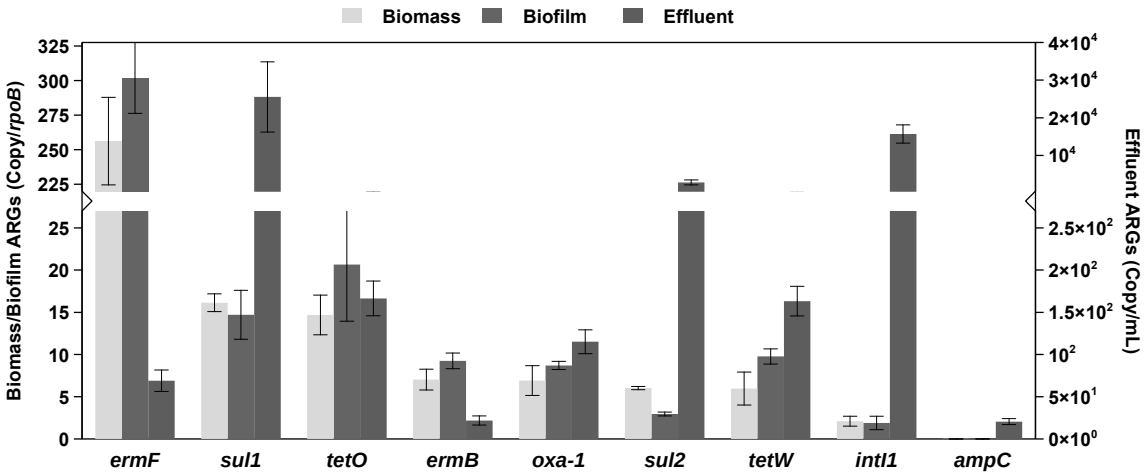


**Figure 2.** Abundance of targeted ARGs in the biomass, biofilm (Copy/*rpoB*) and effluent (Copy/mL) during the addition of 250 (µg/L) sulfamethoxazole. ARGs are sorted by the biomass abundance (highest to the lowest). Bar charts and error bars respectively represent the mean values and standard deviations calculated according to the results from three samples collected on at different days during the addition of 250 (µg/L) sulfamethoxazole and each of their triplicate qPCR results. For biofilm results, mean values and standard deviations were calculated according to the triplicate qPCR results

*ermF* was the most abundant targeted ARG in the biomass and biofilm. Previous work has also identified *ermF* as being among the most abundant ARGs in anaerobic digesters.[38, 39] The predominant ARGs in the biomass sample (250 µg/L sulfamethoxazole) in order of normalized abundance were *ermF*, *sul1*, *tetO*, *ermB*, *oxa-1, sul2, tetW*, *intI1* and *ampC*. Results indicated that *oxa-1*, *ampC*, *tetO*, and *tetW* were all significantly higher in biofilm samples ($p$<0.031) compared to their normalized biomass ARG abundance. These higher abundances in the biofilm could be due to affinity of specific ARGs to the biofilm matrix as well as potential differences in microbial community structure. For example, tetracycline resistance genes have previously been shown to migrate from the water column to drinking water biofilms,[40] while *ampC* has been commonly detected in wastewater biofilms.[2]

Effluent ARG profiles were vastly different from those of the biomass and biofilm. Perhaps most surprisingly, *ermF,* which accounted for approximately 70% of targeted ARGs in biomass and biofilm, contributed only 0.2% in the effluent. The lack of *ermF* presence in the effluent is significant considering that previous work has shown mesophilic anaerobic digestion to enhance the presence of *erm*-type genes.[41] Specifically, it highlights the importance of membrane separation for removal of *ermF*-harboring bacteria in AnMBRs, as well as implies a limited *ermF* presence on extracellular MGEs. The most abundant genes detected in the effluent (when the influent contained 250 µg/L sulfamethoxazole) were *sul1* and *intI1*, followed by *sul2*, *tetO*, *tetW*, *oxa-1*, *ermF*, *ermB,* and *ampC*. Class 1 integrons gene abundance was significantly greater in the effluent relative to the biomass, suggesting the presence of *intI1* on extracellular MGEs. The low relative abundance of *intI1* compared to biomass ARGs is consistent with previous studies on anaerobic digestion.[38, 42]

Recent studies investigating the fate of ARGs in MBR systems have observed similar differences between effluent ARG distributions and those of the reactor biomass, with effluent ARGs also

being dominated by *sul1* in most instances.[12, 21, 26, 43, 44] The significance of *sul1* in membrane-separated effluents is noteworthy, especially given its strong association with class 1 integrons.[45] Previous studies found that effluent ARG abundances spanned a broad range of values (from $10^3$ to $10^7$copies/mL). Our study found ARG abundances on the lower range of that spectrum (all detected ARGs $<10^{4.5}$ copies/mL). This observation, in combination with the significantly lower detection of specific genes (such as *ermF* and *ermB*, $<10^2$copies/mL) as compared to previous studies of MBR and conventional systems,[12] implies that AnMBRs could serve to reduce the overall rates of release of ARGs to the environment.

### 3.3. Most ARGs followed increasing trends in biomass during incremental antibiotic addition

The overall impact of the sequential addition of sulfamethoxazole, erythromycin, and ampicillin at incremental concentrations (10 µg/L, 50 µg/L, and 250 µg/L) on the biomass ARG profile (normalized to *rpoB*) is shown in Figure 3A. Although no overall trends in total ARG abundance were observed across the operational timeframe (i.e., all three antibiotic phases), there were several antibiotic-specific trends for both total and individual ARG abundance.

Sulfamethoxazole

Total biomass ARG abundance significantly increased with the addition of sulfamethoxazole, as well as with each concentration increment from 10 to 250 µg/L ($p<0.05$). Assessment of ARG abundance without the dominant influence of *ermF* (subset Figure 3A) revealed a similar trend, increasing to above 58 copies/*rpoB* at 250 µg/L sulfamethoxazole. Specific genes that increased significantly with sulfamethoxazole concentration were *ermF* and *sul1*. The parallel increase of these two genes in the biomass despite *ermF*'s notably lower presence in the effluent compared to *sul1* may be due to previously observed differences in the genotypes of class 1 integrons and their incorporation of *sul1* and *ermF* genes separately.[46] At the highest concentration of sulfamethoxazole (250 µg/L), both *sul2* and *oxa-1* genes were also observed to increase significantly ($p<0.028$) compared to 50 µg/L, which suggests that a higher threshold of activation may cause the emergence of such genes. *oxa-1* has previously been observed in gene cassettes with both *sul1* and *sul2*, implying that there could be a basis for its enrichment by sulfamethoxazole at high concentrations.[47]

Regarding sulfonamide resistance genes, *sul1* was consistently found at higher concentrations than *sul2* in the biomass ARG profile (Figure 4A). The biomass abundance of *sul1* before antibiotic addition averaged $8.3\pm0.8$ copies/*rpoB* which significantly increased at each incremental concentration of sulfamethoxazole to $16.1\pm1.0$ at 250 µg/L ($p<0.043$), corroborating previously observed correlations for *sul1* in both anaerobic and conventional wastewater treatment systems.[13, 48, 49] Conversely, *sul2*'s relative abundance was $4.5\pm0.7$ without any notable changes at 10 µg/L and 50 µg/L ($p>0.27$). At 250 µg/L, however, *sul2* significantly increased by $37\pm4.1\%$ ($p<0.038$) compared to 50 µg/L. The increased response of *sul1* at lower sulfamethoxazole concentrations could be associated with its common occurrence on small conjugative plasmids, while *sul2* emergence at higher concentrations may highlight its previously observed presence on larger less-mobile plasmids.[50]

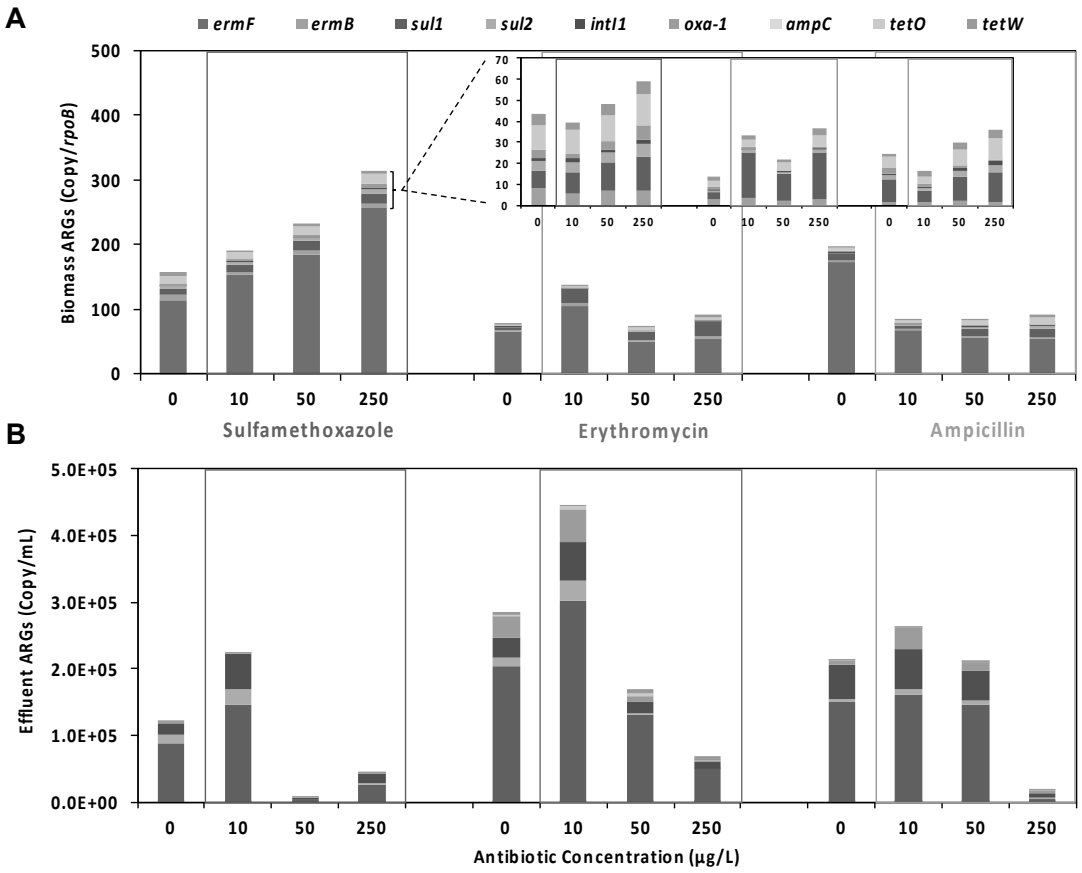


**Figure 3.** (A) Abundance of targeted ARGs (Copy/*rpoB*) in the biomass (inserted diagram shows abundance of targeted ARGs (Copy/*rpoB*) in the biomass excluding ermF gene); (B) Abundance of targeted ARGs (Copy/mL) in the effluent, during the addition of increment concentrations of sulfamethoxazole, erythromycin and ampicillin. Bars represent the mean values of three temporal sampling points (except for 10 µg/L erythromycin and 50 µg/L ampicillin which represent two samples, and 0 µg/L ampicillin which represents one sample) collected during the addition of each increment concentration of antibiotics and each of their triplicate qPCR results.

Erythromycin

Ten days after the conclusion of the sulfamethoxazole addition phase, erythromycin was added to the influent in a replicate concentration sequence. Between the last sulfamethoxazole increment (250 µg/L) and the pre-erythromycin sample, total targeted ARG abundance in the biomass decreased significantly from 315±38 to 79.4±11.1 copies/*rpoB* ($p$<0.001; Figure 3A). This drastic decrease was mainly due to reductions in *ermF* and *sul1* gene abundance. Given that both *ermF* and *sul1* genes in the biomass were positively correlated with sulfamethoxazole concentration, it is likely that ceasing its addition to the influent resulted in a sudden decrease in its selective pressure on the microbial community. The addition of 10 µg/L erythromycin to the influent increased both *sul1* and *ermF* biomass abundance significantly, although no significant change was observed for the remaining ARGs.

The targeted erythromycin-associated ARGs were *ermF* and *ermB*, both of which confer resistance to macrolides by antibiotic target alteration (ribosomal target methylase).[51] Although *ermF* abundance was found to be approximately an order of magnitude higher than *ermB* in the

biomass (Figure 4B), both genes followed a similar trend during addition of erythromycin. Abundances of *ermF* and *ermB* significantly increased by ($p$<0.046) at 10 μg/L erythromycin. However, this increase was then followed by significant decreases ($p$<0.038) at 50 μg/L. No significant changes in *erm* gene abundances were observed between 50 and 250 μg/L. This was somewhat surprising, given previous reports of erythromycin at 250 μg/L initiating resistance in a range of bacterial strains.[52] Although both the current and previous studies have found a lack of correlation between erythromycin concentration and biomass *erm* gene abundances,[53] their significant increase at 10 μg/L implies that the antibiotic can affect total microbial community resistance even at trace levels.

Ampicillin

After the erythromycin phase, total biomass ARGs targeted increased significantly from 91.5±16.3 to 197.6±27.5 copies/*rpoB* ($p$<0.001), primarily due to a rise in *ermF* gene abundance. These observations suggest that the final erythromycin concentration of 250 μg/L had an inhibitory effect on even *ermF*-associated bacteria. During the ampicillin phase, several of the targeted ARGs, including *sul1*, *sul2*, *ampC*, *tetO*, and *tetW*, increased progressively at 50 and 250 μg/L ampicillin ($p$<0.031) (Figure 3A).

The β-lactam resistance genes targeted in this study, *oxa-1* and *ampC*, both encode for enzymatic inactivation of antibiotics as their resistance mechanism. Still, *oxa-1* and *ampC* genes followed remarkably different trends in the biomass ARG profile during ampicillin addition. Although *oxa-1* decreased significantly from 2.46±0.10 copies/*rpoB* before the addition of ampicillin to 1.1±0.1 at 10 μg/L ($p$=0.042), it remained relatively constant thereafter at 50 and 250 μg/L. *ampC*, however, significantly increased at each increment of ampicillin (Figure 4C). This trend might be due to the fact that *ampC* and *oxa-1* genes belong to different molecular classes of β-lactam resistance genes: *ampC* directly confers resistance to ampicillin whereas *oxa-1* confers resistance to cloxacillin and oxacillin. Overall, the biomass ARG profiles of this study revealed that the abundances of ARGs, including those conferring resistance to the same antibiotic, can follow vastly different trends in relation to increasing antibiotic concentrations (e.g., *oxa-1* vs. *ampC*). Although the cause of this phenomenon is unclear, it may relate to specific gene presence on plasmids, plasmid mobility and conjugation, and microbial community dynamics. However, given the consistent reactor biomass concentration (MLVSS), the short duration of antibiotic phases in relation to the system SRT (>300 d), and the stability of microbial communities typically observed in AnMBRs,[23, 54, 55] it is unlikely that microbial abundance dynamics alone could have resulted in such high variability in ARG copy number (which ranged from several- to over 10-fold). Therefore, this is an indication of the importance of ARG presence on plasmids and their subsequent horizontal transfer across and/or loss from the microbial community.

### 3.4. 10 μg/L antibiotic concentrations induced spikes in total effluent ARG abundance

As discussed in section 3.3, the ARG profile of the AnMBR effluent was distinct from that of the biomass. Due to the potential bias associated with extracellular ARGs in the effluent (post-membrane filtration), ARG abundances were normalized to volume (mL) for the analysis of all effluent samples.

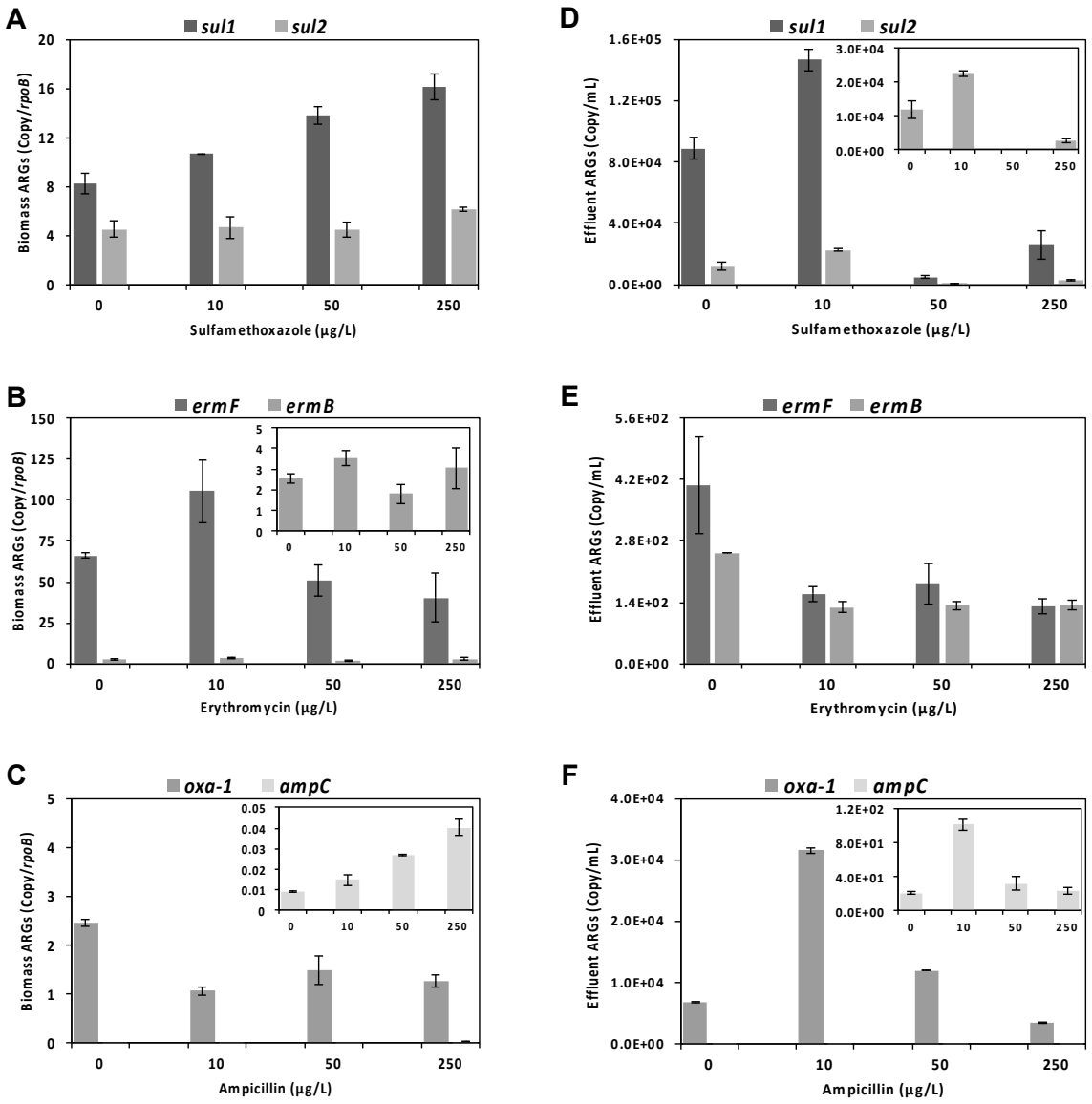


**Figure 4**. Abundance of antibiotic corresponding genes in biomass (Copy/*rpoB*) and effluent (Copy/mL) when (A) and (D) sulfamethoxazole; (B) and (E) erythromycin; (C) and (F) ampicillin was added to AnMBR. Inserted diagrams were used to magnify the genes with low abundance. Bars and error bars represent the mean values and standard deviations, respectively, of three temporal sampling points (except for 10 µg/L erythromycin and 50 µg/L ampicillin which represent two samples, and 0 µg/L ampicillin which represents one sample) collected during the addition of each increment concentration of antibiotics and each of their triplicate qPCR results.

Sulfamethoxazole

Effluent ARG profiles during sulfamethoxazole addition were dominated by *sul1*, *sul2*, and *intI1*. The addition of 10 µg/L sulfamethoxazole to the influent significantly increased the abundance of *sul1*, *sul2*, *intI1*, and *oxa-1* genes ($p$<0.026). However, at 50 µg/L sulfamethoxazole, a sharp decrease in the abundance of all ARGs ($p$<0.007) except for *ermF* and *ermB* caused a reduction in

total effluent ARG abundance (Figure 3B). The abundance of the same ARGs (*sul1*, *sul2*, *intI1*, *oxa-1*, *tetO*, and *tetW*) grew significantly ($p$<0.034) when sulfamethoxazole was increased from 50 to 250 μg/L, although only to approximately 20% of the 10 μg/L levels.

Both sulfonamide resistance genes (*sul1* and *sul2*) followed a similar trend in the effluent across the varying sulfamethoxazole influent concentrations (Figure 4D). For both, the addition of 10 μg/L resulted in abundance significantly increasing by 68.5±1.1% and 83.3±2.5% ($p$<0.005), respectively. *sul1* and *sul2* abundance then dramatically decreased by about 2 orders of magnitude, at 50 μg/L and finally increased again slightly at 250 μg/L sulfamethoxazole ($p$<0.012).

The dominance and common trends of the *sul*-type genes in the effluent, along with *intI1* and *oxa-1*, are a strong testament to their likely presence on a single gene cassette encoded for recombination by *intI1*.[45] Gene cassettes combining *sul1*, *sul2*, and *oxa-1*, specifically, are well documented and have been found in several wastewater-associated pathogenic bacteria.[47] Although the abundant presence of such a cassette-carrying plasmid in AnMBR effluent is cause for further scrutiny, this observation must also be put in perspective. For example, anaerobic systems have been shown to harbor a significantly lower abundance of *intI1*-associated ARGs (including *sul* and *tet* genes) than activated sludge.[49, 56] Although not well understood, the reason for this lower ARG abundance may be associated with faster rates of ARG loss by bacteria under anaerobic conditions.[57]

Erythromycin

The abundance of total ARGs in the AnMBR effluent before commencement of the erythromycin run (0 μg/L erythromycin), in contrast to the biomass ARG profile, increased approximately six-fold relative to the last stage of sulfamethoxazole addition (Figure 3B). This spike in ARGs could have been created by a sudden die-off of a subset of resistant bacteria previously selected for by sulfamethoxazole at 250 μg/L and the subsequent release of their intracellular DNA. Alternatively, it is possible that the membrane cleaning performed prior to the erythromycin phase reduced the propensity of the membrane biofilm matrix to partially or fully retain plasmids/extracellular ARGs. This scenario seems likely, especially considering observations of recent studies documenting the importance of biofilms in MBR systems for achieving higher removals of ARGs.[22, 44] Effluent ARGs increased further at the first erythromycin concentration of 10 μg/L, then gradually decreased at subsequent concentrations of 50 and 250 μg/L.

During erythromycin addition, both *ermF* and *ermB* genes were more abundant in the effluent than in the previous run and followed a parallel trend to each other (Figure 4E). However, a significant decrease was observed in total abundance of both *ermF* and *ermB* by 60±7.8% and 48±1.3% ($p$<0.027), respectively, at 10 μg/L erythromycin. Subsequent increases in erythromycin concentration to 50 μg/L and 250 μg/L did not significantly change the abundance of *ermF* or *ermB* in the effluent ($p$>0.471). Overall, *erm* gene abundance dynamics were entirely independent of those of the dominant ARGs in the effluent, as was the case during the sulfamethoxazole run. The lack of correlation between these macrolide resistance genes and the remaining ARGs is likely attributable to their minor contribution to the effluent profile (<0.2% of total ARGs), which implies their lack of extracellular presence in the biomass and/or presence on MGEs of a size range large enough to be rejected by the membranes.

Ampicillin

Similar to the interim period between sulfamethoxazole and erythromycin addition, ARG abundance increased significantly prior to ampicillin addition (Figure 3B). Total effluent ARGs increased slightly after the addition of ampicillin at 10 μg/L then decreased to original abundance

at 50 μg/L. When the influent ampicillin concentration was increased to 250 μg/L, however, a drastic drop by approximately an order of magnitude was observed in total effluent ARG abundance, led by *sul1*, *sul2*, *intI1*, and *oxa-1* ($p$<0.032). Considering the low concentrations (<4 μg/L) of ampicillin detected in the AnMBR effluent, this drop may have been due to a significant reduction in extracellular plasmid-associated ARGs in the biomass.

As previously observed for the sulfamethoxazole- and erythromycin-associated resistance genes, *oxa-1* and *ampC* (conferring β-lactam resistance) followed a parallel trend in their effluent ARG abundance during the ampicillin run despite a lack of consistency between the two genes in their corresponding biomass samples (Figure 4F). The highest abundance of *oxa-1* and *ampC* in the effluent occurred at 10 μg/L ampicillin, which was about 3.7 times higher than at 0 μg/L for both genes. *oxa-1* then significantly decreased by 61.9±0.7% and 66.3±1.2% at 50 and 250 μg/L ($p$<0.001), respectively, while *ampC* decreased significantly by 68±4.3% ($p$<0.001) at 50 μg/L.

Overall, a common trend of increase at 10 μg/L and subsequent decrease at 50 and 250 μg/L was observed for most ARGs in the effluent during all three antibiotic phases (Figure 3B). There are two probable explanations for this phenomenon. First, it is possible that the initial trace level exposure to the antibiotics increased horizontal gene transfer. This could have led to a temporary increase in extracellular plasmid DNA, which thereby amplified the harboring of plasmid-based resistance within the biomass microbial community for the remainder of individual antibiotic phases. If, after initial antibiotic exposure, the rates of horizontal gene transfer then slowed, this would have again reduced the levels of extracellular plasmid-based ARGs and lowered the rate of ARGs passing through membranes into the effluent. Second, it is likely that the cleaning of AnMBR membranes prior to commencement of each antibiotic run reduced the effect of membrane biofilm-based ARG removal, which then gradually increased along with the development of sub-critical fouling layers.[22]

### 3.5. AnMBR effluent ARB largely unaffected by different antibiotics and concentrations

The results of heterotrophic plate counts (HPC) and ARB plate counts (including sulfamethoxazole, erythromycin, ampicillin, and tetracycline) revealed a lack of consistent trends between antibiotic concentration and specific ARB effluent abundance, both in terms of their absolute and HPC-normalized values (Figure S2). One exception was in the case of sulfamethoxazole resistant bacteria, which increased in relation to total bacterial count with the increasing influent sulfamethoxazole concentrations. A recent study by Le et al. on an MBR system similarly found that among 19 antibiotics targeted, sulfamethoxazole was one of three antibiotics that increased with its corresponding ARB.[58] It is important to note that the heterotrophic enumeration methodology used conventionally (and in this study) does not accurately account for anaerobic microorganisms.

The overall highest counts among targeted ARB in the effluent were erythromycin resistant bacteria. Sulfamethoxazole and ampicillin resistant bacteria were lower and generally close to one another in terms of CFU/mL, while tetracycline resistant bacteria had the lowest absolute plate counts. The dominant abundance of erythromycin resistant bacteria in the effluent stood in contrast to a relatively minor presence of erythromycin-associated ARGs, implying that their persistence was achieved through alternative macrolide and/or multidrug resistance genes than those detected in the AnMBR biomass.

### 3.6. Correlation between antibiotics, ARGs, and ARB indicated the presence of multi-drug resistance

Correlation analysis of the effluent revealed that all ARB types targeted were strongly correlated with each other (ρ>0.79, $p$<0.011; Figure 5), implying that a large fraction of effluent bacteria may have harbored multidrug resistance. However, sulfamethoxazole resistant bacteria were the only ARB that showed significant positive correlation to their corresponding antibiotic (ρ=0.72, $p$=0.041). Strong correlation between the abundance of some ARB and their corresponding ARGs were also observed, such as ampicillin resistant bacteria and both *oxa-1* (ρ=0.84, $p$=0.005) and *ampC* (ρ=0.93, $p$<0.0001) genes. This observation might suggest that, in the AnMBR effluent, a considerable portion of *oxa-1* and *ampC* genes were predominantly located within bacterial cells. The lack of correlation between other ARB in the effluent and their corresponding ARGs (i.e., sulfamethoxazole and erythromycin resistant bacteria) highlights one of the main limitations of this study and qPCR-based ARG targeting in general: any conclusions drawn from this analysis are only representative of the predetermined gene targets used. The inclusion of more and/or different ARGs could lead to significantly different results in terms of total ARG abundance and their relative distributions. Metagenomics-based approaches have previously been developed for ARG screening in wastewater systems and could serve to circumvent such limitations in future studies.[59, 60]

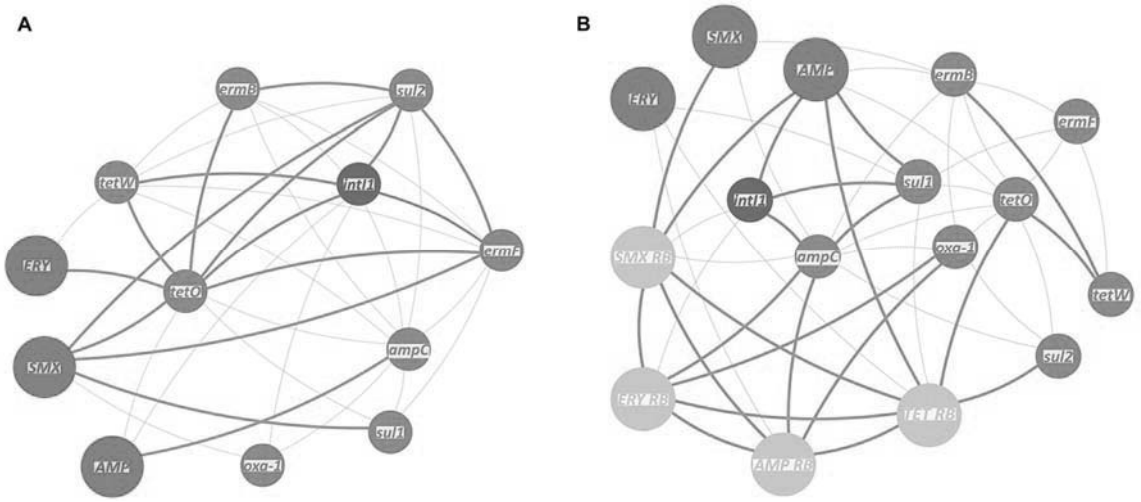


**Figure 5.** Network analysis representing the correlations between antibiotics concentration (pink circles), ARGs (teal circles), class 1 integrons (blue circles) and ARB (yellow circles) (A) in biomass; (B) in effluent. SMX, ERY, AMP and TET stand for sulfamethoxazole, erythromycin, ampicillin and tetracycline; respectively. RB also stands for resistant bacteria. A solid connection shows strong, significant correlations (ρ > 0.7 or ρ < -0.7; and p<0.05), and a dashed line represents weak correlations (0.3 < ρ < 0.7 or -0.7 < ρ < -0.3; and p<0.05)

The analysis also demonstrated that there were a high number of correlations among ARGs in the biomass (such as *sul2* and *ermB*) and comparatively fewer among ARGs in the effluent (such as *sul1* and *ampC*) (Figure 5). Abundance of the integrase gene *intI1* in the biomass was strongly correlated with *ermF* (ρ=0.72), *sul2* (ρ=0.71), *tetO* (ρ=0.79), and *tetW* (ρ=0.71) genes ($p$<0.0001), while it was not significantly correlated with *sul1* ($p$>0.35). In effluent samples, however, *intI1* was only correlated with *sul1* (ρ=0.9, $p$<0.0001) and *ampC* (ρ=0.7, $p$=0.0001) genes. Given the frequent association of *sul1* with class 1 integrons,[45] this correlation between *sul1* and *intI1* in the effluent is not surprising. Such integrons' ability to initiate the recombination of a variety of ARGs in

associated cassettes,[45] however, is an indication of the potential presence of additional undetected ARGs. Further, the association of class 1 integrons (encoded for by *intI1*) with horizontal gene transfer in anaerobic enviroments[61, 62] suggests a relatively consistent MGE-based ARG occurrence in the AnMBR biomass. Although this may be expected given the high density of anaerobic microorganisms in the bioreactor, the lack of consistency between biomass and effluent *intI1*-ARG correlations implies that only a fraction of these MGEs are being released into the effluent.

Membrane separation in MBRs has been shown to be a key factor in the reduction of effluent ARGs.[11, 58] Such improved removal of ARGs is likely attributable to their predominant presence within bacterial cells, as extracellular plasmids have previously been shown to be completely permeable to 0.1 µm membranes.[63] However, given that ARG-associated plasmids can reach sizes exceeding 90 kb,[64] it is likely that such large plasmids would be at least partially retained by microfiltration membranes when present in "supercoiled" form.[65] This, in combination with the reduction in effective pore size caused by membrane biofilm formation in MBRs,[22, 44, 66] could conceivably lead to significant variability in the removal of even extracellular ARGs by the AnMBR. Such variability would, indeed, contribute to the differences observed in this study between the biomass and effluent ARG profiles. The phenomenon responsible for this variability (i.e., physical retention of extracellular plasmid-located ARGs) could also serve to accentuate the advantages of AnMBR systems for ARG reduction by providing an additional barrier to ARG release. Evaluating ARG-associated plasmid retention via membrane separation is an important topic for future research.

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