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Effects of influent municipal wastewater microbial community and antibiotic resistance gene profiles on anaerobic membrane bioreactor effluent

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ABSTRACT

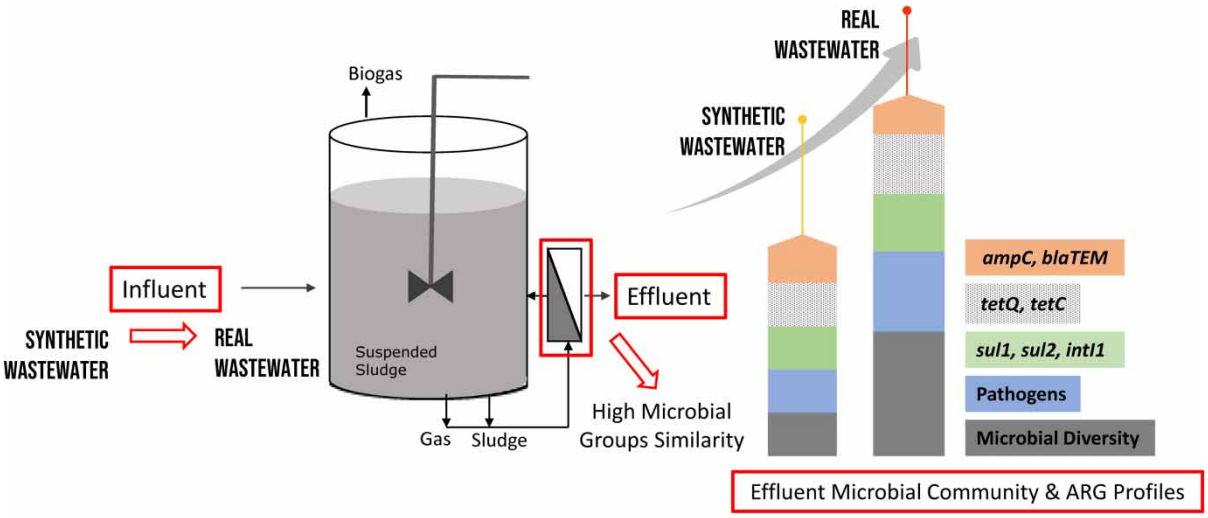
Municipal wastewater management is an important target area for reducing the spread of antibiotic resistance, especially given the parallel increasing need for water reuse. Anaerobic membrane bioreactors (AnMBRs) have the potential to play a key role in safely expanding non-potable wastewater reuse practices. In the present study, the effect of commencing treatment of municipal wastewater by an AnMBR was evaluated after an extended startup phase using only synthetic wastewater. Antibiotic resistance genes (ARGs) associated with sulfonamides, tetracyclines, and β-lactams were quantified, and effluent microbial community progression was analyzed. Results indicated that the AnMBR effluent inherently harbored all targeted ARGs prior to the introduction of real wastewater (10^4–10^9 copies/100 mL effluent). *sul1*, *sul2*, and *intI1* genes were notably higher initially than other genes and markedly increased after the system was transitioned to municipal wastewater. Although potentially pathogenic bacteria made up over 20% relative abundance of the influent, AnMBR effluents showed a marginalization of these groups as their microbial communities more closely resembled the tightly bound layer of membrane biofilms. This work highlights the need for emerging treatment systems to be evaluated on a basis that incorporates the differentiation of system-associated ARGs and assesses their environmental transmission potential within the effluent communities.

Key words: AnMBR, antibiotic, ARGs, biofilm, domestic wastewater, pathogen

HIGHLIGHTS

- Assessments were made pre- and post-municipal wastewater introduction to an AnMBR.
- ARGs conferring resistance to three antibiotic classes were evaluated in effluents.
- Changes in microbial communities across the AnMBR system were interpreted.
- Influent wastewater ARG and microbial profiles induced changes in the AnMBR effluent.

GRAPHICAL ABSTRACT



INTRODUCTION

The global propagation of antibiotic resistance is rapidly increasing the threat of pathogenic bacterial infections becoming essentially untreatable in clinical settings. It is evident that antibiotic resistance occurs in both natural and engineered environments, even when the selective pressure of antibiotics is not present (Davies & Davies 2010). This reality has led to extreme challenges with respect to devising risk assessment models and regulatory frameworks for managing antibiotic resistance proliferation (Berendonk *et al.* 2015). One of the critical aspects of antibiotic resistance management that can be utilized to alleviate this eminent threat is the limiting of the spread of resistance elements in wastewater treatment systems (Pruden *et al.* 2013). Given the biological nature of wastewater treatment, it is important to understand the naturally occurring background resistance within both current and proposed treatment systems.

When designing new wastewater treatment systems, antibiotic resistance and public health goals need to be managed in concert with overall improved system sustainability (Pruden 2014). Wastewater treatment sustainability involves both the treated product's reusability and the energy balance associated with the treatment process itself (Grant *et al.* 2012). From this perspective, the anaerobic membrane bioreactor (AnMBR) is an emerging technology that combines high-quality effluents with sustainable energy recovery potential (Shoener *et al.* 2016). AnMBRs utilize an anaerobic digestion system in combination with membrane separation, which produces a particle-free permeate with its nutrient content retained. This allows for methane recovery during treatment while also producing an effluent that is highly suitable for non-potable reuse (Wu & Kim 2020). As such, AnMBRs have also been investigated for their potential to alleviate effluent risks associated with contaminants of emerging concern (Monsalvo *et al.* 2014; Fox & Stuckey 2015; Zhang *et al.* 2021). To this end, the removal rates and removal mechanisms of such contaminants in AnMBRs have been shown to be vastly different from both aerobic MBRs and non-membrane-based anaerobic treatment systems (e.g., up-flow anaerobic sludge blankets) (Ji *et al.* 2020; Lim *et al.* 2020).

From the perspective of microbial risk, AnMBRs have been shown as possessing specific advantages toward improving pathogen removal from effluents during the treatment of municipal wastewaters (Harb & Hong 2017a). Recent studies have indicated that they are capable of achieving pathogen and indicator organism reduction levels that would allow for direct effluent reuse in agricultural irrigation (Harb & Hong 2017b; Peña *et al.* 2019). This can serve to alleviate the need for downstream disinfection and, consequently, the risk of the formation of disinfection byproducts that are typically exacerbated in nutrient-rich wastewater effluents (Wang *et al.* 2007a; Zhang *et al.* 2011). Still, antibiotic resistance dynamics in effluent reuse systems have yet to be effectively linked to regulatory guidelines. This is largely due to the prevailing reality that the effect of antibiotic resistance elements, such as antibiotic resistance genes (ARGs), on the persistence and infectivity of pathogens in effluents is not well understood.

Antibiotic-resistant bacteria (ARB) and ARG release from wastewater treatment plants is, in part, a result of the activity of the functional microbial communities within those plants (Rizzo *et al.* 2013). ARGs in influent wastewaters play a key role in shaping the respective resistance profiles of treatment systems, but the nature of individual unit processes (i.e., aerobic, anoxic, and anaerobic) has been shown to significantly impact the nature of the ARGs they predominantly harbor (Tong *et al.* 2019). Based on this, it is important to understand how emerging treatment technologies, such as the AnMBR, behave with respect to antibiotic resistance element dissemination through their effluents.

A number of recent studies have aimed at addressing antibiotic resistance in AnMBRs. Various aspects of ARG dynamics in these systems have been assessed including the presence and concentration of various antibiotics and micropollutants (Harb *et al.* 2016; Zarei-Baygi *et al.* 2019), membrane fouling effects on effluent ARGs (Cheng & Hong 2017; Zarei-Baygi *et al.* 2020b), differences between sludge biomass and effluent ARGs (Zarei-Baygi *et al.* 2020a), the removal of ARGs from AnMBR effluents by UV/H_2O_2 treatment (Augsburger *et al.* 2021), the impact of adding an electrochemical system to an AnMBR (Li *et al.* 2021), and the effects of co-treatment of multiple waste sources (Lou *et al.* 2020). Despite the significant advancements achieved through the above-mentioned efforts, only three studies to date have investigated ARG removal rates in AnMBRs treating real municipal wastewater sources (Kappell *et al.* 2018; Lou *et al.* 2020; Augsburger *et al.* 2021). As such, these studies were able to provide insights into the apparent log removal values (LRVs) of various ARGs across AnMBR systems. The remaining investigations so far have assessed ARG dynamics without the selective pressure of ARGs or ARB in the influent (i.e., by utilizing synthetic wastewater). Concomitantly, such investigations showed that ARGs are emitted from AnMBR systems even if operated for extended periods of time with no ARGs in their influent. Still, no research to date has attempted to differentiate between the intrinsic ARG abundances of AnMBR effluents and the concentrations contributed by the wastewater influent. Such background investigation is highly necessary in order to effectively interpret apparent ARG LRVs in AnMBRs.

In the present study, we examine the effluent ARG and microbial community profiles of an AnMBR immediately before and during the initial operation of the system with real municipal wastewater influent. The AnMBR utilized for this work was started up and operated for 8 months on a synthetic feed prior to its exposure to municipal wastewater. ARG profiles in the influent and the effluent were compared, and the changes in effluent concentrations were elucidated over a 42-day period. Changes in the system's microbial community abundances were estimated using 16S rRNA gene-based high-throughput sequencing, with a specific focus on potentially pathogenic groups.

MATERIALS AND METHODS

AnMBR configuration and operation

The AnMBR consisted of a lab-scale continuously stirred tank reactor (CSTR) with a 3-L working volume (Chemglass Life Science, USA) operating at 35 °C. Two external cross-flow membrane units, defined as membrane A and membrane B, were used to house flat sheet polyvinylidene difluoride (PVDF) microfiltration membranes of 0.2 μm pore size (Microdyn Nadir, Germany) and an effective area of 0.057 m^2 each (Figure 1). The system was operated with an average transmembrane flux of 6.7 ± 0.6 L/m^2-h. The hydraulic retention time (HRT) and the sludge retention time (SRT) were 33 h and 360 days, respectively. To prevent fouling, the membranes were continuously sparged through headspace biogas recirculation to scour membrane surfaces, relaxed for 60 s every 30 min, and backwashed for 20 min daily. The influent treated was municipal wastewater collected from two local treatment plants in the Keserwan and Batroun Districts in Lebanon with chemical oxygen demand (COD) ranging from 529 to 630 mg/L, resulting in an organic loading rate of 0.37–0.44 g/L-d. The measured water quality parameters of the influent are summarized in Table 1. The influent was stored at 4 °C prior to treatment. The reactor was originally seeded with sludge obtained from an anaerobic digester treating food waste and agricultural waste streams originating from the Bekaa region in Lebanon. From AnMBR startup until the introduction of municipal wastewater (Day 1), the system was fed with synthetic wastewater with composition as shown in Table S1 (Supplementary Material, Appendix A) for a period of 8 months. Membranes were harvested for biofilm samples on Days 21 and 42 of the experiment for the evaluation of their microbial communities.

Water quality and biogas testing

The influents and effluents were monitored for water quality by testing COD, total suspended solids (TSS), volatile suspended solids (VSS), volatile fatty acids (VFAs), phosphate, sulfate, ammonia, total nitrogen, and nitrate contents. The COD was measured in accordance with the USEPA Reactor Digestion Method followed by colorimetric determination on a Hach

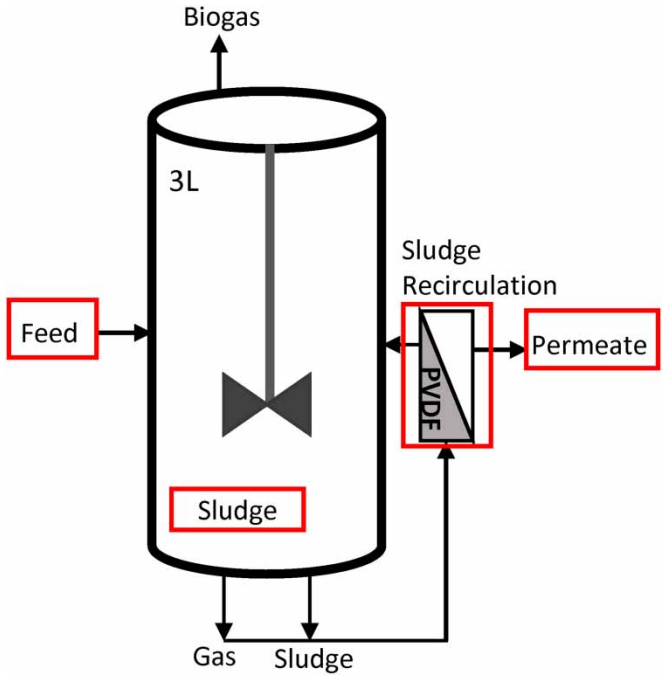


Figure 1 | A schematic representation of the lab-scale AnMBR. Sampling points are indicated by red squares and include (1) influent, (2) anaerobic sludge, (3) membrane, and (4) AnMBR effluent. Please refer to the online version of this paper to see this figure in colour: http://dx.doi.org/10.2166/wrd.2022.018.

Table 1 | Influent characteristics of the municipal wastewater used in the study

Influent characteristics	Concentration (mg/L)
Chemical oxygen demand (COD)	578 ± 68
Suspended solids (SS)	166 ± 4
Total nitrogen (TN)	70.5 ± 0.7
NH_3-N	56.6 ± 0.5
NO_3^--N	<0.1
NO_2^--N	<0.1
SO_4^{2-}	13.4 ± 1.3
PO_4^{3-}	5.5 ± 1.3

DR3900 Spectrophotometer. TSS and VSS were tested following APHA Standard Method 2540 (Baird *et al.* 2005). NH_3-N and total nitrogen concentrations were determined using Test 'N Tube high range kits, methods 10031 and 10072, respectively. VFAs (acetate, propionate, and butyrate), phosphate, sulfate, and nitrate were quantified by ion chromatography (882 Compact IC Plus) equipped with a conductivity detector and the 858 Professional Sample Processor (Metrohm AG, Switzerland). The influent and effluent samples were filtered using Nylon 0.2 μm syringe filters. VFAs were run on a Metrosep Organic Acids – 250/7.8 (6.1005.200) column at 0.5 mL/min, and the remaining parameters were measured on a Metrosep A Supp 5 – 250/4.0 (6.1006.530) column at 0.7 mL/min.

Biogas production from the AnMBR was quantified by sampling from the reactor headspace, and samples were stored in 1-L Tedlar bags prior to analysis. Methane content was determined using an Agilent 7890B gas chromatograph with thermal conductivity detection (GC-TCD) with the oven set at 90 °C and the detector at 250 °C. Dissolved methane in the permeate was also measured by using the headspace method. Briefly, 30 mL of the effluent sample was collected in a syringe and then

transferred to a 250 mL flask filled with nitrogen. The flask was shaken for 1 min, heated for 10 min, and then shaken again briefly to strip the dissolved methane into the gas phase. The gas sample in the flask was measured for methane content by GC-TCD.

Biomass sampling and microbial community characterization

Samples from influents, effluents, reactor biomass, and membranes were collected for DNA extraction and stored at −20 °C. Influent and effluent samples were filtered on 0.45 μm mixed cellulose ester (MCE) circular membrane filters (Millipore, USA). The effluent sample prior to real wastewater introduction was initially taken (Day 1), followed by effluent samples taken on Days 10 and 42 for comparison purposes. Reactor biomass samples of 2 mL were taken from the suspended sludge and centrifuged at 12,000 rpm for 10 min for pellet retention. AnMBR membrane biomass samples were taken when harvesting the membranes, cut into equal sections of 4.75 cm × 3 cm, and suspended in RNAprotect Reagent (Qiagen, USA). The membrane biomass samples were divided into loosely and tightly bound, respectively. The loose biofilm portion was gently scraped along the surface of the membrane and collected into a 2 mL tube. For the same membrane segment, the tightly bound layer was equally cut into strips and collected into a 2 mL tube. DNA was extracted for all samples using the DNeasy PowerSoil Kit (Qiagen, USA) according to the manufacturer's protocol. Extracted DNA concentrations and qualities were measured on a Nanodrop ND 1000 spectrophotometer Version 3.3.0.

For the characterization of bacterial and archaeal microbial communities, the V4 region of the 16S rRNA gene was targeted for amplification using universal primers 515F and 806R. The amplicons were multiplexed and sequenced on the Illumina NovaSeq 6000 platform with paired-end 250 bp read lengths by Novogene Genomics (Singapore). FASTQ files generated were analyzed on the mothur bioinformatics platform (Schloss *et al.* 2009) based on the Schloss Miseq SOP (Kozich *et al.* 2013). The UCHIME algorithm was used to filter sequences that were aligned by the SILVA reference database (Quast *et al.* 2012). Operational taxonomic unit-based clustering was used with an average neighbor algorithm at a 0.03 cutoff limit. The Ribosomal Database Project (RDP) classifier database with the 16S rRNA gene Training Set (Version 18) was employed for taxonomical classification to the genus level (Wang *et al.* 2007b). Representative species were identified using the BLASTN algorithm on the National Center for Biotechnology Information (NCBI) database (Altschul *et al.* 1990). Relative abundances (RAs) were calculated at the genus level and for representative species based on normalization to total sequences per sample.

ARG quantification

Extracted DNA from influent and effluent samples were used to establish their associated ARG profiles by quantitative PCR (qPCR). The ARGs targeted in this study were chosen to represent genes that are commonly found in wastewater and that confer resistance to multiple antibiotic classes, including sulfonamides (*sul1* and *sul2*), β-lactams (*ampC* and *bla-TEM*), and tetracyclines (*tetC* and *tetQ*), as well as a class 1 integron-integrase gene (*intI1*) (Chopra & Roberts 2001; Chen & Zhang 2013; Zhou *et al.* 2018). To estimate total cell counts in the influent and effluent, the *rpoB* gene was quantified based on its ubiquitous presence as a single-copy gene in bacteria (Dahllöf *et al.* 2000). qPCR standards were initially prepared through the amplification of target genes by PCR from a pool of real wastewater samples' extracted DNA and the excising of appropriate gel bands after electrophoresis for use as standard. qPCR was conducted on a CFX Connect Real-Time PCR Detection System (BioRad, USA) using the Forget-Me-Not qPCR Master Mix (Biotium, USA). Amplicon specificity was determined by melt curve analysis through increasing temperatures from 65 to 95 °C at increments of 0.5 °C. Additional details on PCR and qPCR procedures are provided in the Supplementary Material (Appendix A). Thermocycling conditions used for qPCR for all targeted genes are shown in Table S2 of Supplementary Material, Appendix A along with amplicon sizes and primer sequences.

RESULTS AND DISCUSSION

Performance of the AnMBR

The AnMBR showed consistent performance after the introduction of municipal wastewater as its treated influent. This was an indication that, after several months of startup on synthetic wastewater, the system was able to transition to real wastewater without a lapse in effluent quality or biogas production. All operating parameters, including temperature, transmembrane flux, HRT, organic loading rate (OLR), and TSS/VSS, were maintained at a steady state throughout the experiment (Table 2). At the permeate flux employed of 6.7 ± 0.6 L/m^2-h, transmembrane pressure (TMP) remained lower than 37 kPa with no significant trends across the duration of the experiment. COD removal was in the range of 86.5 ± 3.2%

Table 2 | Summary of AnMBR operational parameters and performance

Operational parameter	Value
Temperature	35 °C
Transmembrane flux	6.7 ± 0.6 L/m^2-h
HRT	33 h
OLR	0.39 ± 0.3 g/L-d
VSS	4.9 ± 0.7 g/L
VSS/TSS	0.83 ± 0.05
Performance parameter	Value
COD removal	86.5 ± 3.2%
CH_4 production	290 ± 12 mL/g COD
Biogas CH_4 content	78 ± 6%
Effluent CH_4	13–35 mL/L effluent
Effluent CH_4 oversaturation ratio	0.93–2.25

and was stable throughout all days of operation. No notable removal was observed for ammonia, total nitrogen, or phosphate, as can be expected for an anaerobic unit process-based system.

Biogas collected from the reactor headspace was consistent with the average OLR of the system, with methane recovered accounting for 290 ± 12 mL/g COD removed. The methane content of the biogas was also relatively high at 78 ± 6% of total biogas produced, with the remaining fraction being contributed by carbon dioxide. Due to the relatively low OLR of the system, this biogas methane fraction was in line with what is expected for the treatment of low-strength wastewaters by AnMBRs (Hu *et al.* 2020). As the potential for COD removal by sulfate reduction was negligible (<2%) and no significant changes in reactor VSS were observed, the remaining COD removed was likely also predominantly converted to methane but discharged with the reactor effluent. Based on an application of Henry's law for the operating temperature of the reactor and percent methane in the headspace, the solubility of methane would occur at a rate of approximately 15 mL/L effluent. It has been previously shown, however, that methane concentrations in effluents can exceed this theoretical rate of solubility due to oversaturation (Chen *et al.* 2018).

To investigate the potential oversaturation of methane in the AnMBR permeate, actual methane concentrations were measured throughout the experiment. Interestingly, a notable difference in the oversaturation rate was observed between the period prior to the first membrane harvesting (Days 1–21) and the period after the placement of a new membrane until the end of the experiment (Days 22–42). For samples taken between Days 1 and 21, the effluent methane oversaturation was measured at 1.23 ± 0.20, while between Days 22 and 42, the average oversaturation values were significantly higher (at 2.05 ± 0.17). Given a lack of any notable differences in TMP between those two periods, a likely explanation for this observation is a higher occurrence of methanogenic activity in the membrane biofilms themselves. This has been previously implicated as a cause for oversaturation, as methanogens in the membrane biofilm layer can discharge methane directly into the reactor permeate (Smith *et al.* 2015). The occurrence of this phenomenon in the current experiment was corroborated by a higher RA of methanogens (predominated by *Methanothrix*) in the tightly bound biofilm layer of replicate membrane samples taken on Day 42 *versus* those taken on Day 21 (Figure S1, Supplementary Material, Appendix A). Considering that methanogens were essentially absent from the influent samples treated (<0.02% RA), the potential influence of introducing municipal wastewater to the system on this increase in methanogens in the later stages of the experiment is not clear. Still, it is possible that the physical and/or chemical parameters of the influent allowed membrane biofilm development that facilitated higher methanogenic presence and activity. Given that the effluent methane concentrations observed exceeded saturation, dissolved methane recovery to mitigate greenhouse gas emissions is a critical point for consideration. Among the methods that can be used for biogas recovery from AnMBR effluents, micro-porous membranes, PDMS membranes, and downflow hanging sponge reactors have shown promise as effective solutions (Crone *et al.* 2016; Sanchis-Perucho *et al.* 2020). A more recent study also demonstrated the possibility of enhanced effluent methane recovery through a novel vibrating AnMBR model, in which a vibrating membrane promoted dissolved methane recovery (Wang *et al.* 2022).

Microbial diversity increased after the introduction of municipal wastewater

The high-throughput sequencing results of 16S rRNA gene amplicons for samples of influent wastewater, AnMBR effluent, reactor biomass, tightly bound membrane biofilms, and loosely bound membrane biofilms were utilized to conduct both α-diversity and β-diversity analyses. To assess the microbial similarity between different sample types, a principal coordinate analysis (PCoA) was performed using a Theta-YC similarity distance matrix (Figure 2). AnMBR microbial communities were also evaluated for diversity based on richness, evenness, and the Shannon diversity index (Table 3). Figure 2 results showed that, overall, AnMBR microbial communities increased in diversity after the introduction of municipal wastewater. Still, the effluent communities remained closely clustered to pre-municipal wastewater exposure conditions while also showing high similarity to the tightly bound membrane biofilms.

PCoA indicated that AnMBR microbial communities were notably separated from municipal wastewater samples based on the dominant principal coordinate (PC1, 40.97% loading). For the purpose of validating the microbial community distribution of the wastewater treated in this study (represented by influent Days 1 and 17), three other wastewater samples taken within the same timeframe of the study were also analyzed in comparison to all samples. Results of the analysis indicated that the alternative wastewater samples were largely comparable to the wastewater samples treated in the study based on PC1. Similarly, all AnMBR samples (reactor biomass, membrane biofilms, and effluents) were closely clustered on PC1 while being significantly separated from influent samples. A notable separation between AnMBR samples was also observed based on the secondary principal coordinate (PC2, 15.63%). This separation generally showed that AnMBR effluents exhibited the highest similarity to the tightly bound membrane biofilms, as compared to reactor biomass and loosely bound membrane biofilm samples. These results suggest that the microbial compositions of AnMBR effluents are directly impacted by the makeup of the tightly bound membrane biofilm layers that exist directly upstream of the permeate and are only indirectly affected by influent wastewater communities.

In parallel to the notable microbial differences between AnMBR samples and the influent wastewater treated, a calculation of diversity metrics for microbial richness (R), evenness (E), and the Shannon Index (H') generally showed a notable increase in diversity across AnMBR samples after the introduction of real wastewater to the system. Influents were highest among all

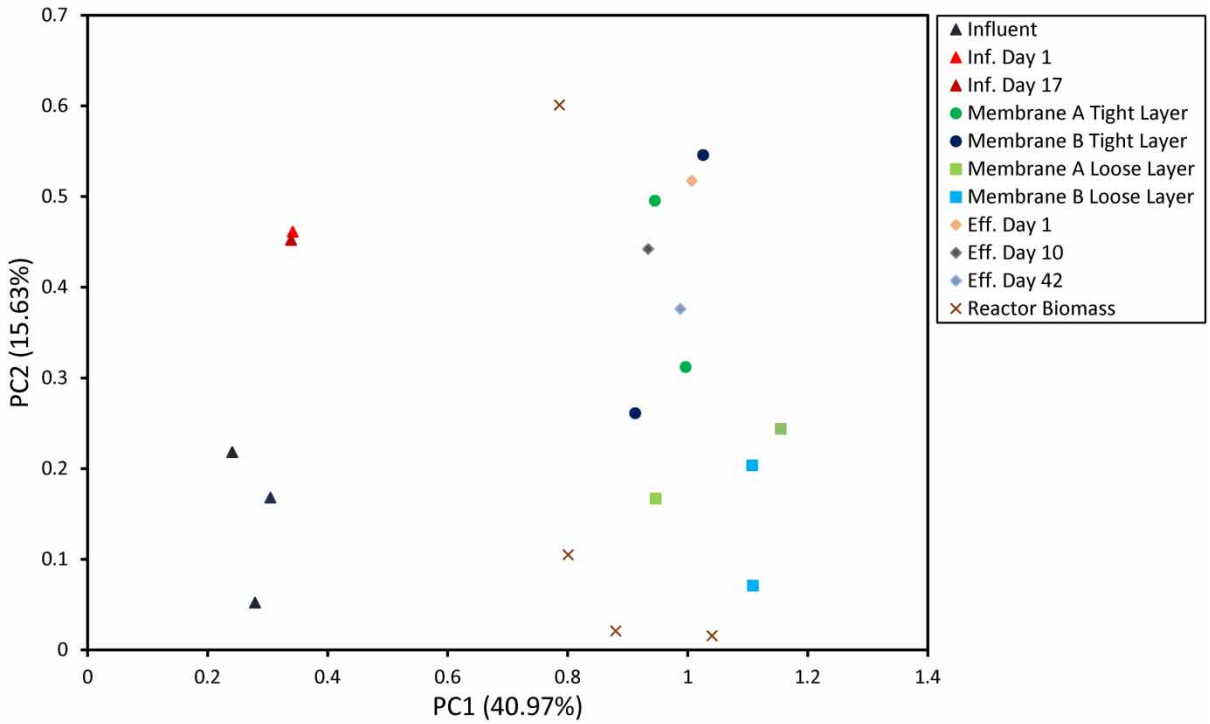


Figure 2 | PCoA plot of microbial abundance similarity for AnMBR samples using a Theta-YC similarity distance matrix for genus-level sequence clustering. Eff. Day 1 represents the effluent sampled before real wastewater introduction, whereas Eff. Day 10 and Eff. Day 42 represent effluent samples taken during real wastewater treatment.

Table 3 | Summary of microbial α-diversity metrics for different sample types

	Index		
Sample	**Richness (*R*)**	**Evenness (*E*)**	**Shannon (*H′*)**
Influent Day 1	1,595	0.60	3.73
Influent Day 17	1,748	0.62	3.87
Reactor biomass Day 3	1,521	0.60	3.71
Reactor biomass Day 10	1,704	0.57	3.55
Reactor biomass Day 17	1,676	0.59	3.70
Reactor biomass Day 42	1,717	0.53	3.27
Effluent Day 1	1,151	0.55	3.44
Effluent Day 10	1,157	0.55	3.45
Effluent Day 42	1,548	0.59	3.64
Membrane TL Day 21	1,138	0.55	3.43
Membrane LL Day 21	1,121	0.48	3.02
Membrane TL Day 42	1,597	0.59	3.66
Membrane LL Day 42	1,545	0.57	3.52

Note: TL indicates the membrane tight layer and LL indicates the membrane loose layer. Microbial richness was calculated based on the rarefaction curve analysis at a uniform sequence count cutoff of 74,000 reads.

samples based on *R*, *E*, and *H′* values, with only reactor biomass samples from the later stages of the experiment exhibiting similarly high richness values (ranging from 1,676 to 1,717). Interestingly, the increase in *R* among reactor biomass samples was accompanied by a notable decrease in *E* and *H′* over the timeframe of the experiment. This is an indication that the introduction of real wastewater to the system, although concurrently driving the presence of a higher number of operational taxonomic units (OTUs) within the reactor sludge, also reduced the diversity of the sludge in terms of the evenness of the distribution of the microbial groups present. An analysis of reactor biomass microbial community RAs on Days 3, 10, 17, and 42 of the experiment elucidated some of the key microbial groups responsible for the decrease in evenness observed (Figure S2). For example, notable increases in both unclassified Bacteroidetes and unclassified Syntrophaceae were seen in all reactor biomass samples taken after Day 3, with RAs increasing from 6–7 to 15–20% for each of the two groups. These increases corresponded with significant decreases in unclassified Azonexaceae, *Azonexus*, and *Pseudomonas*. These observed changes in the suspended sludge's relative composition represented a shift in the dominant fermentative and short-chain fatty acid degrading bacterial groups, which were likely driven by the introduction of a more complex substrate (municipal wastewater) that favored the presence of syntrophic groups such as Syntrophaceae (Zhang *et al.* 2020; Zhao *et al.* 2020).

Membrane biofilm microbial communities also showed significant increases in richness for the membranes harvested on Day 42 of the experiment as compared to those harvested on Day 21 (both tightly bound and loosely bound layers). Inversely to the reactor biomass, however, *E* and *H′* values for the membrane biofilms increased with prolonged exposure to real wastewater. This indicated that richness and evenness were correlated for the biofilm-based anaerobic communities and (unlike for reactor biomass) that the biofilm microbial communities were not as predominated by specific groups, as richness increased after municipal wastewater introduction. Similar to membrane biofilms, the AnMBR effluent microbial communities also increased in overall diversity toward the end of the experiment, with the effluent sample of Day 42 showing notably higher *R*, *E*, and *H′* values as compared to those of Days 1 and 10. Despite the rise in diversity observed for AnMBR samples across the experiment (including those of the reactor permeate), the dominant microbial groups of the effluent remained relatively stable after the introduction of municipal wastewater (as indicated by PCoA).

AnMBR effluents marginally impacted by influent microbial communities

In order to elucidate the possibility of relevant microbial communities of the influent persisting through the AnMBR system, a targeted analysis was performed on OTUs representing potentially pathogenic groups (Figure 3). The dominant microbial groups of the AnMBR effluent were also correspondingly evaluated. The *rpoB* gene was used as a marker to estimate bacterial

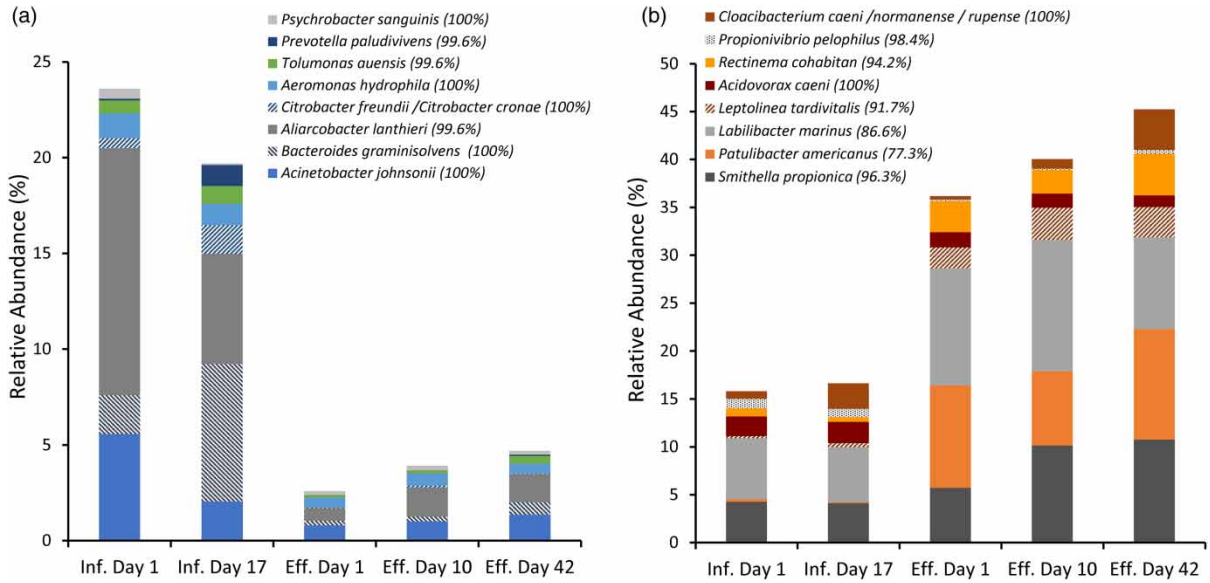


Figure 3 | The relative abundance (%) of microbial community OTUs in the influent wastewater and AnMBR effluent samples for (a) OTUs associated with potentially pathogenic species and (b) other dominant OTUs with a relative abundance of greater than 1% in the effluent.

counts in the influent and effluent of the AnMBR (Figure 4). Overall, results showed that potentially pathogenic groups had low RAs in the AnMBR effluent as compared to the influent but increased mildly after the introduction of municipal wastewater. In addition, *rpoB* copy numbers in the effluent notably increased (by over an order of magnitude) after the system was exposed to the influent microbiome.

OTUs most closely related to potentially pathogenic groups that were found in the influent with an RA of 1% or greater included those associated with *Psychrobacter*, *Prevotella*, *Tolumonas*, *Aeromonas*, *Citrobacter*, *Aliarcobacter*, *Bacteroides*, and *Acinetobacter* spp. (Figure 3(a)). For comparison, reference wastewaters (not treated in this study) were also compared

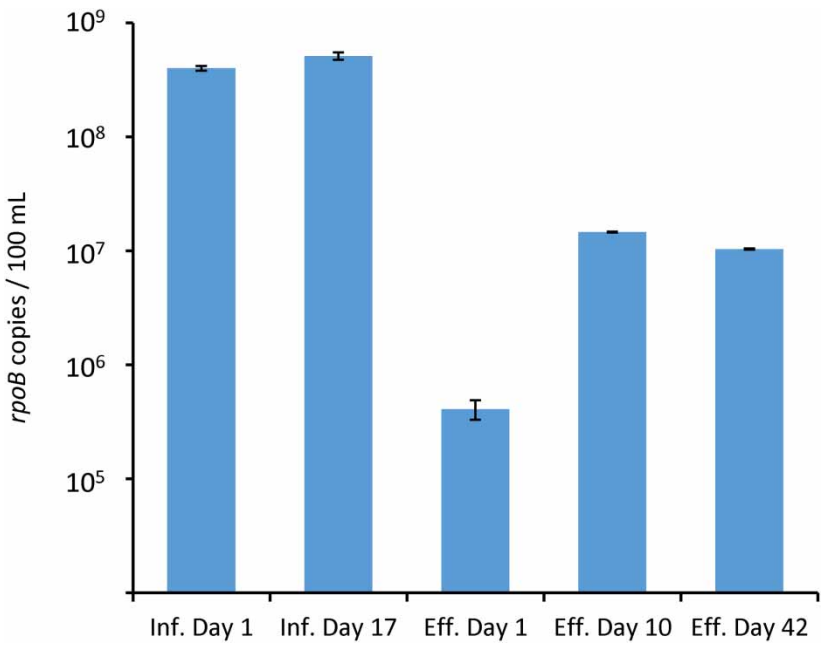


Figure 4 | *rpoB* gene copy abundance detected in the influent wastewater and AnMBR effluent samples.

to the influent of the AnMBR. Results of the comparison indicated that these pathogen-associated OTUs were essentially ubiquitous in influents of the region, with cumulative RAs ranging from 20 to 30% of total sequences from five wastewater samples compared (Figure S3, Supplementary Material, Appendix A). The AnMBR effluent harbored markedly lower levels of these groups. Prior to the exposure of the system to municipal wastewater, the total RA of the aforementioned OTUs made up 2% of sequences but progressively increased to nearly 5% RA throughout the experiment. These results suggest that although the AnMBR could significantly reduce the levels of potentially pathogenic bacteria, the discharge of such groups in the effluent can still be directly impacted by the microbial composition of treated wastewater influents.

Still, effluent microbial communities of the AnMBR were predominated by several groups that were consistently present throughout the experiment. Such groups included *Cloacibacterium*, *Propionivibrio*, *Rectinema*, *Acidovorax*, *Leptolinea*, *Labilibacter*, *Patulibacter*, and *Smithella*. *Labilibacter*, *Patulibacter*, and *Smithella*-associated OTUs dominated the effluent, collectively consistently making up over 30% of RAs (Figure 3(b)). These groups have generally been shown as associated with AnMBR system biomasses (both suspended and attached) (Inaba *et al.* 2020; Chen *et al.* 2021), which suggests that their presence in the effluent may be associated with a preferential capacity to permeate the microfiltration membranes used for sludge separation. The consistent predomination of such microbial groups in the effluent is likely to suppress the potential for other influent-associated microbial groups to regrow in the treated wastewater discharges and, thus, reduce pathogen-associated risks.

Although it is generally expected for the membranes of MBRs (including AnMBRs) to reject bacteria based on pore size exclusion, studies have shown that a fraction of bacteria can end up passing through membranes and seeding effluent microbial communities based on several physical factors. Among such factors are absolute membrane pore size (as compared to the nominal pore size), the deformability of bacterial cells and their TMP-induced passage through membrane pores, and the regrowth of microbes in effluents based on residual substrate and nutrient availability (Harb & Hong 2017b; Yu *et al.* 2017; Zarei-Baygi *et al.* 2020b). As such, previous work that utilized heterotrophic plate counts (HPCs) to determine total and antibiotic-resistant bacterial abundances in AnMBR effluents found that cell totals ranged from 10^4 to 10^6 colony-forming units (CFUs)/mL of the effluent for a system treating synthetic wastewater (Zarei-Baygi *et al.* 2019). Given that HPCs inherently favor only a minor fraction of total bacteria (Van Nevel *et al.* 2017), total bacterial abundances in effluents may be significantly different from those detectable by conventional methods.

To estimate total bacterial counts in the AnMBR's discharged permeate, the *rpoB* gene was employed based on its presence as a universal single-copy genome-associated bacterial marker. Results of qPCR-based quantification showed that *rpoB* abundance was in the range of 10^8–10^9 copies/100 mL of the municipal wastewater influent treated (Figure 4). The AnMBR effluent taken on Day 1 indicated that, prior to exposure to municipal wastewater, *rpoB* abundance existed in the range of $<10^6$ copies/100 mL of the effluent. After the introduction of municipal wastewater as the influent, however, a marked increase in *rpoB* abundance was apparent, with abundance in the effluent increasing by over an order of magnitude to approximately 10^7 copies/100 mL. Despite this increase, overall LRVs for the *rpoB* gene by the system remained at around 1.5 or higher throughout the experiment. If taken as an approximation of total bacterial count, the LRV for the *rpoB* gene suggests that the reduction rate of potentially pathogenic bacteria is even greater than that of total bacterial cells (with LRVs ranging between 2 and 2.5).

ARGs varied in apparent removal level by the AnMBR

ARGs representing genes that confer resistance to sulfonamides, tetracyclines, and β-lactams were targeted in the influent and effluent of the AnMBR. Samples were taken to differentiate between background ARG abundances from the AnMBR system itself and the influence of the ARG/microbial profiles of the influent. Based on this, AnMBR effluent ARGs were quantified on Day 1 of the experiment immediately prior to the transition from synthetic wastewater to municipal wastewater, in addition to on Days-10 and 42. Results of this quantification indicated that the ARGs targeted were generally present in the AnMBR effluent prior to the introduction of municipal wastewater but at varying levels of abundance (ranging from 10^4 to 10^9 copies/100 mL) (Figure 5).

The quantification of ARGs in the influent municipal wastewater showed that target genes were present at concentrations ranging from 10^7 to 10^{11} copies/100 mL. Sulfonamide resistance genes, *sul1* and *sul2*, were highest with both genes at abundances over 10^{10} copies/100 mL influent. Tetracycline resistance genes, *tetC* and *tetQ*, were found at concentrations above 10^8 and 10^9 copies/100 mL, respectively. bla_{TEM} was found at a similar abundance as the targeted *tet* genes, while ampC was lowest in the municipal wastewater at 10^7–10^8 copies/100 mL. In addition, high levels of the class 1 integron-integrase

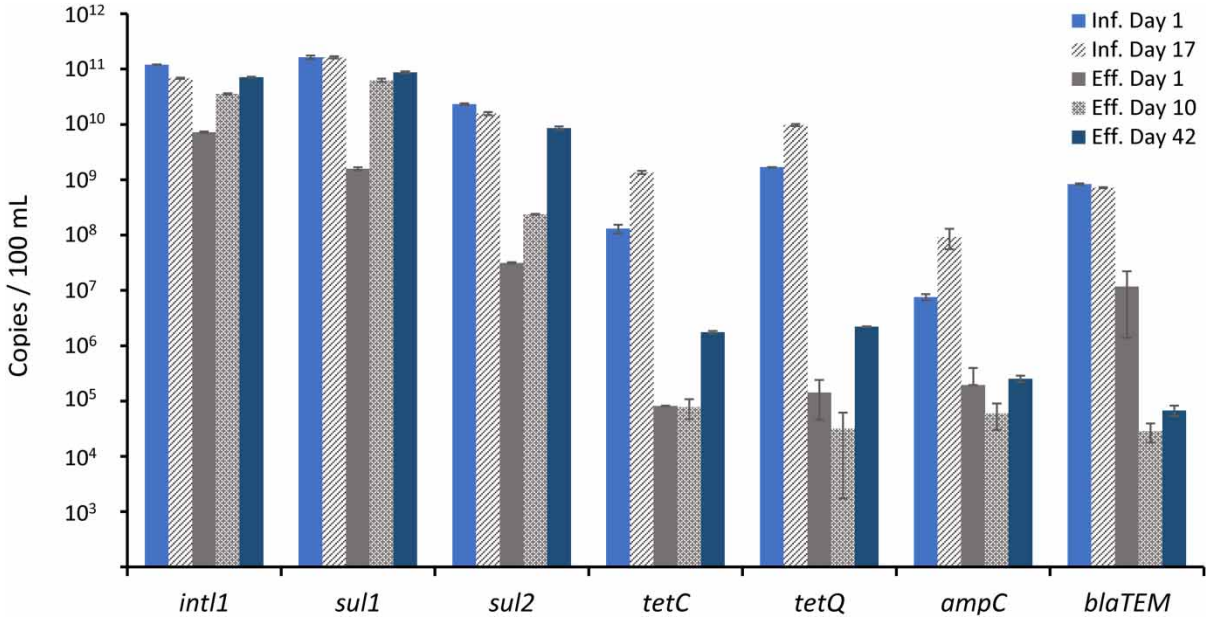


Figure 5 | ARG and *intI1* gene copy abundances detected in the influent wastewater and AnMBR effluent samples.

gene, *intI1*, were detected (up to 10^{11} copies/100 mL), indicating the widespread occurrence of mobile genetic elements in the influent microbial communities (Barlow *et al.* 2004).

In the effluent of the AnMBR system, ARG abundances were affected differently across the different target genes by the introduction of municipal wastewater after Day 1. *sul1*, *sul2*, and *intI1* all followed a similar trend by significantly increasing between Days 1 and 42 of the experiment (Figure 5). The increase of these three genes in parallel is likely due to the co-occurrence of the sulfonamide resistance genes on plasmids containing the *intI1* gene (Frank *et al.* 2007; Wu *et al.* 2010). Their corresponding increase over time, in combination with the relative stability of the effluent's dominant microbial communities, suggests that the introduction of municipal wastewater led to the dissemination of *sul1* and *sul2* into the effluent microbial communities through horizontal gene transfer. This ultimately led to *sul1* and *sul2* gene copy numbers in the effluent being on nearly the same order of magnitude as was found in the influent.

Tetracycline-associated resistance genes, *tetC* and *tetQ*, were consistently found at much lower abundances in the effluent as compared to *sul* genes but also as compared to their corresponding influent concentrations. On Days 1 and 10, *tetC* and *tetQ* abundances were similar and in the range of 10^4–10^5 copies/100 mL. Both of their abundances, however, increased by an order of magnitude on Day 42 to above 10^6 copies/100 mL. Although these tetracycline-associated ARGs predominantly exist within chromosomes, their transfer between bacteria through their presence on conjugative transposons has also been reported (Chung *et al.* 1999; Shi *et al.* 2021). Based on this, it is possible that, after an initial exposure period, an increase in transfer events led to the observed increases in *tetC* and *tetQ*. Still, the apparent LRV for these genes by the AnMBR was at or above 3.0 and was vastly higher than for the sulfonamide resistance genes in this study.

Results for ARGs conferring resistance to β-lactams were similar to those of *tetC* and *tetQ*. The overall apparent LRV for *ampC* was in the range of 2.0–2.5, while for bla_{TEM}, the LRV was above 4.0 for the samples taken during the municipal wastewater treatment period. Although *ampC* and bla_{TEM} were present in the influent municipal wastewater at markedly higher concentrations than the AnMBR effluent, the effluent profile did not show any significant increase in these genes over the course of the experiment. This suggests that any abundance of *ampC* and bla_{TEM} genes that were detected in the effluent can be considered from the AnMBR microbiome itself and essentially uninfluenced by the influent ARG content. Although these ARGs (among various others that confer resistance to β-lactams) have been broadly shown as transferrable horizontally through plasmids in the water environment (Singh *et al.* 2018; Zarei-Baygi & Smith 2021), there may have been limited selective pressure toward the propagation of their associated plasmids (as compared to those that likely harbored *sul1* and *sul2*).

Previous studies that examined ARGs in AnMBRs have found effluents to contain abundances comparable to those reported in the present study, with various overlapping target genes detected in the range of 10^4–10^8 copies/100 mL (Kappell

et al. 2018; Zarei-Baygi *et al.* 2019; Lou *et al.* 2020; Zarei-Baygi *et al.* 2020b). Effluent ARG abundances in these studies were generally within a similar scale of each other regardless of whether the influent wastewater being treated was synthetic or from a microbially active source (i.e., municipal wastewater). This suggests that the AnMBR microbiome is the primary source of ARG discharge through effluents to the environment, regardless of whether an ARG loading exists from the influent. In further accordance with the present work, sulfonamide-associated resistance genes (*sul1* and *sul2*) have been shown as dominant in effluents even when influent wastewaters harbor these genes at equivalent concentrations to other target ARGs. Despite the relative independence of ARG profiles of AnMBR effluents from their treated sources, the results of the present work showed that certain genes (including *sul1*, *sul2*, *tetC*, and *tetQ*) can increase in AnMBR effluents when introduced to municipal wastewater ARG loading. Further, the variations of specific genes appeared to be relatable based on the antibiotic class to which they confer resistance. This, in combination with the noted trends of potentially pathogenic groups in the AnMBR effluent, is an indication that the correlation between ARG abundances and the bacterial groups that can harbor and/or acquire them (through horizontal gene transfer) is a key area that requires further study. ARG profiles of the suspended biomass and membrane biofilms should also not be overlooked in future work on AnMBRs treating real wastewater sources, especially considering that previous studies (treating synthetic wastewater) have found biomass and effluent ARG profiles to have notably different trends (Zarei-Baygi *et al.* 2020a).

CONCLUSIONS

Although AnMBRs possess great potential for the treatment of municipal wastewaters in terms of energy recovery, emerging contaminant removal, and permeate quality, their role in reducing ARG and pathogen proliferation remains understudied. In the present work, inherent *versus* influent-induced microbial community and ARG profiles were interpreted for the treatment of municipal wastewater by an AnMBR. Results suggested that the introduction of a real (microbially active) wastewater influent after extended operation with synthetic wastewater led to an increase in microbial diversity throughout the system. Despite a high abundance of potentially pathogenic groups in the influent, the effluent microbiome remained relatively stable in its microbial composition. ARG abundances also appeared to be primarily driven by the AnMBR's existing microbial makeup, although certain ARGs (including those associated with sulfonamide resistance) notably increased in abundance after the introduction of municipal wastewater. Overall, this work highlights the importance of considering the association of ARGs with specific wastewater treatment technologies – in addition to their influents – so as to devise strategies that can effectively reduce their proliferation in the environment.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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