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Anaerobic membrane bioreactors (AnMBRs) have the potential to significantly improve upon the efficiency of conventional wastewater treatment. However, emerging microbial contaminants, such as antibiotic resistance genes, remain understudied in these systems. The present study shows that influent antibiotics can alter both microbial communities and ARG profiles of AnMBR effluents, which can affect downstream spread of ARGs to potentially pathogenic bacteria.

# Microbial community and antibiotic resistance profiles of biomass and effluent are distinctly affected by antibiotics addition to an anaerobic membrane bioreactor

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## Abstract

The transfer of antibiotic resistance to pathogenic bacteria is one of the most eminent human health threats and a concern in water reuse schemes. Anaerobic membrane bioreactors (AnMBRs) are an emerging wastewater treatment biotechnology that have significant potential for mainstream wastewater treatment. However, AnMBR effluents remain largely unexplored with respect to their microbial community composition and their antibiotic resistance profiles. In this study, we operated a bench-scale AnMBR for the treatment of domestic wastewater containing antibiotics (250 μg/L each of sulfamethoxazole, ampicillin, and erythromycin) and evaluated microbial community structure and antibiotic resistance gene (ARG) dynamics in both the biomass and effluent. Results showed that ARG abundances in the biomass of the AnMBR consistently increased throughout the experiment, while the effluent ARG abundances saw a sharp increase upon initial antibiotics exposure to the system and then dropped immediately thereafter. Further, a vastly more variable microbial community was observed in the AnMBR effluent as compared to the biomass. Several potentially pathogenic genera in the effluent were strongly correlated with the abundance of specific resistance genes (e.g., *sul1*), as well as a class 1 integrase gene (*intI1*). Overall, results of this study provide useful insights into the association of ARGs with microbial community dynamics in AnMBR, which is needed to devise operational and design strategies to lessen dissemination of antibiotic resistance to the environment.


Key words: antibiotic resistance; microbial community; pathogenic bacteria; anaerobic membrane bioreactor

## 1. Introduction

Antibiotic resistance is an issue of crucial concern as one of the most imminent human health risks. In the US, antibiotic resistance is currently responsible for over $20 billion in excess health costs and 8 million additional hospital days.[1] The proliferation of antibiotic resistance in the environment and clinical settings is dictated by the dissemination of antibiotic resistance genes (ARGs).[2] Resistance spreads due to the selective pressures raised by antimicrobial compounds and through vertical and horizontal gene transfer (VGT and HGT) mechanisms among and across different bacterial populations, respectively. Recent studies have revealed a considerable increase in the number of bacterial species capable of resisting different antibiotic classes.[1, 3, 4]

Wastewater treatment plants (WWTPs) are known reservoirs of ARGs. Studies have revealed that variable concentrations of various ARGs are released daily from WWTPs to the environment, sometimes at levels that can even exceed those observed in influent wastewater.[5] Although wastewater can be considered a resource of energy, water, and nutrients, recovering these resources must be balanced with protecting the downstream environment, including mitigating emerging threats such as antibiotic resistance.[6] Anaerobic membrane bioreactors (AnMBRs) are an emerging biotechnology that can recover energy from wastewater via biogas production and reduce residuals production, while also potentially playing a role in lessening antibiotic resistance dissemination.[7-9] Given that the biomass of WWTPs contains both a broader range and higher concentrations of ARGs than do WWTP effluents,[4] the vastly lower biomass production of AnMBRs as compared to conventional aerobic WWTPs has the potential to significantly reduce the overall release of ARGs to the environment. Further, microorganisms and larger mobile genetic elements

(MGEs) can be effectively retained in the reactor via the membrane barrier and its associated biofilm layer, reducing dissemination of antibiotic resistance in the effluent.[8]

Despite these potential advantages, the fate of antibiotics and ARGs in AnMBRs remains understudied. In a previous study, we demonstrated that individual influent antibiotics at a range of concentrations (10 to 250 μg/L) can significantly alter the abundance of both related and unrelated ARGs in the biomass and effluent of AnMBRs.[8] It has been reported elsewhere that the impact of a mixture of antibiotics on ARG profiles can be considerably greater than an individual antibiotic.[10, 11] Therefore, in the present study we investigated the influence of a mixture of antibiotics on the ARG profile of both the biomass and effluent of an AnMBR. Although no study to date has been able to verify the reasons for variation of ARG profiles in the presence of antibiotics,[12, 13] possible explanations could include HGT,[12, 14] changes in microbial community abundance,[11, 15] or some combination of the two. HGT is known to be a critical factor for dissemination of antibiotic resistance in the environment[14] and is of particular concern in WWTPs: namely, ARGs can spread via HGT from non-harmful bacteria to more virulent pathogenic species, thus posing a serious human health risk. In addition to HGT, the fate of ARGs during wastewater treatment is also intricately connected to microbial community dynamics. Although previous studies have demonstrated relationships between biomass microbial communities and their accompanying ARG profiles during the anaerobic digestion of sewage sludge[16], no studies to date have investigated such associations in mainstream anaerobic wastewater treatment systems like AnMBRs. Further, the effluent of AnMBRs may contain resistant pathogenic bacteria, posing a serious health risk in water reuse schemes. Therefore, a comprehensive investigation of associations between microbial community dynamics and ARGs in AnMBR effluents is needed. Consequently, in the present study, both the microbial communities and

the ARG profiles of the biomass and effluent of an AnMBR were investigated before, during, and after the addition of three antibiotics from different classes.

## 2. Materials and methods

### 2.1. Configuration of bench-scale AnMBR

Detailed information of the bench-scale AnMBR has been reported in our previous study.[8] Briefly, the AnMBR consisted of a continuously stirred-tank reactor (Chemglass Life Science, Vineland, NJ) with a working volume of 5 L and three separate microfiltration silicon carbide membrane modules (Cembrane, Denmark) submerged in the reactor. The effective membrane area of each module was approximately 0.015 $m^2$ and the membrane pore size was 0.1 μm. The AnMBR was seeded with sludge from a mesophilic anaerobic digester at the Joint Water Pollution Control Plant (Carson, CA). The AnMBR was operated at 25°C and fed with a synthetic wastewater representative of domestic wastewater in the US (ESI Table S2).[17] The experiments of the present study commenced two month after the end of the previous study. Membrane modules were chemically cleaned both after the end of the prior experiment and before the commencement of the current study's experiment. Between cleanings, the AnMBR was continuously operated with no antibiotics being added to the influent. Steady-state performance of the AnMBR was reached by the end of this period (defined as consistent COD removal of > 85%, stable biogas production and methane content of > 60% over at least two weeks of operation). Five days after confirming steady-state operation, three antibiotics that included sulfamethoxazole (SMX, a sulfonamide), erythromycin (ERY, a macrolide), and ampicillin (AMP, a β-lactam), were simultaneously added to the influent of the AnMBR at a concentration of 250 μg/L each for a period of one month. Although the estimated total antibiotic concentration in domestic wastewater is around 50 μg/L,[18] a higher non-lethal concentration[11] was used in the present study to

emphasize the antibiotic selective pressure impact on microbial community. The present study was divided into three periods: pre-antibiotics (defined as the time after steady performance was reached and before antibiotic addition), antibiotics loading, and post-antibiotics. To monitor the performance of the AnMBR, mixed liquor suspended solid (MLSS), mixed liquor volatile suspended solid (MLVSS), chemical oxygen demand (COD), biogas production, and methane content of biogas were measured continuously during the experimental period, as described previously.[8] Additional details on the system and operational parameters were also described previously.[8]

**2.2. Quantification of ARGs by qPCR**

Quantitative polymerase chain reaction (qPCR) was performed to quantify targeted ARGs using procedures described previously.[8] For biomass ARG profiles, 2 mL of mixed liquor was collected biweekly, centrifuged, and decanted. For effluent ARG profiles, 50 mL of permeate was freeze-dried using a lyophilizer (FreeZone 2.5 Liter Freeze-Dryer, Labconco, Kansas City, MO). Both biomass and effluent samples, were then stored at -80°C prior to DNA extraction. DNA extraction was conducted using the Maxwell 16 Blood DNA Purification kit (Promega, Madison, WI), recommended by the manufacturer for wastewater sludges, according to manufacturer instructions. qPCR was performed using a LightCycler 96 (Roche, Basel, Switzerland) targeting 8 ARGs commonly found in domestic wastewater[19, 20] including genes conferring resistance to sulfonamides (*sul1* and *sul2*), macrolides (*ermB* and *ermF*), β-lactams (*ampC* and *oxa-1*), and tetracycline (*tetO* and *tetW*), as well as a class 1 integrons-associated gene (*intI1*). Due to variable operon copy numbers of 16S rRNA in bacteria, a single copy molecular marker gene (*rpoB*) was selected for ARG normalization to avoid this bias[21]. Details of thermal cycling and primers for each targeted gene were provided in ESI Table S3.

**2.3. Quantification of antibiotics by LC-MS**

Ten mL of influent and effluent samples were filtered through 0.2 μm PTFE syringe filters (Whatman, GE Healthcare, UK) and then stored at 4°C for no more than 3 days prior to analysis. Antibiotics quantification was performed using direct injection liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS) on a 6560 Ion Mobility Quadrupole Time-of-Flight (IM-QTOF) LC-MS system (Agilent Technologies, Santa Clara, CA) using 1290 Infinity UHPLC, Dual Agilent Jet Stream (ASJ), and EclipsePlus C18 column (2.1 mm; 50 mm; 1.8 μm). Details on method development and calibration protocols are provided in ESI.

**2.4. Microbial community analysis**

Biomass and effluent extracted DNA samples were sent for sequencing at the Microbial Systems Molecular Biology Laboratory (University of Michigan, Ann Arbor, MI) where library preparation and sequencing was performed on the Illumina MiSeq platform using the MiSeq Reagent Kit V2 (2x250 bp reads). To amplify 16S rRNA gene targets, a universal 16S rRNA gene primer set targeting the V4 region was used as described previously.[22] High-throughput sequencing results were then analyzed using Mothur v.1.42.1, with Silva 132 reference database for alignment and Ribosomal Database Project (RDP) reference taxonomy for classification (rarefaction curves are provided in ESI Fig. S1). A non-metric multidimensional scaling (NMDS) plot was conducted to present the distance between samples. To evaluate the richness and evenness of samples, the Inverse Simpson index was calculated for each operational phase to serve as a diversity index.

**2.5. Data analysis**

Analysis of molecular variance (AMOVA)[23] was employed to determine the statistical significance of temporal changes in the microbial community data set. Unweighted principal component analysis (PCA) was conducted using XLSTAT to compare the distance of different

ARG profiles. To determine the significance of linear relationships between ARG abundances and microbial community profiles, Spearman's correlation was conducted using MAXSTAT Pro 3.6 over a 95% confidence interval. Strong positive correlation was determined based on P-value and Spearman's coefficients (ρ) where $p < 0.05$ and $\rho > 0.7$.

## 3. Results and Discussion

### 3.1. AnMBR system performance was robust during antibiotics addition

The AnMBR was operated at a hydraulic retention time (HRT) of 16 h and solids retention time (SRT) of 300 d. Permeate flux was 7 L/m$^2$h (LMH) and transmembrane pressure (TMP) remained lower than 25 kPa throughout the experimental period. MLSS and MLVSS were relatively constant at 10.6 ± 1.3 and 9.6 ± 0.3 g/L, respectively. Total COD in the influent of the AnMBR averaged 453 ± 32 mg/L. COD removal was 90.0 ± 1.8% throughout operation, with antibiotics addition having no significant effect on removal rate. Total biogas production and methane content were also stable during the experimental period, averaging 736 ± 20 and 536 ± 14 mL/d , respectively (ESI Fig. S2a). Details of COD mass balance in the reactor were provided in ESI Fig. S6.

Performance of the AnMBR with regards to antibiotics removal in the effluent (ESI Fig. S2b) was similar to a previous study[8] which explored separate addition of the same antibiotics (AMP and SMX removal range of 89-98% and 69-78%, respectively). One exception was for the case of ERY, which had a slightly lower removal rate in the present study than previously observed (removal of 40-58% versus 67-88% in the previous study). In the previous study antibiotics were added individually to the AnMBR and at incremental concentrations. Thus, it is possible that the simultaneous addition of SMX, ERY, and AMP at higher concentrations (250 μg/L) from the first day of the experiment in the present study resulted in less effective ERY biotransformation in the system. This may have occurred due to the microbial community

191 requiring longer acclimation time under the elevated mixed antibiotics conditions.
192 Erythromycin has shown both higher variability and lower removal rates among mainstream
193 anaerobic treatment systems, in general, as compared to the other antibiotics used in this
194 study.[24]

195 **3.2. AnMBR biomass was dominated by different ARGs than the AnMBR effluent**

196 Biomass ARGs were normalized against the *rpoB* gene (as gene copies/*rpoB*), while,
197 due to the potential significance of extracellular ARGs, effluents were normalized against
198 volume (as gene copies/mL). Despite differing normalization strategies, biomass and effluent
199 ARG profiles and temporal trends were markedly distinct (Fig. 1). The only exception was day
200 46 (post-antibiotics period), where the abundance of all effluent ARGs increased significantly.
201 The increase in effluent ARGs after the antibiotics loading period could be due to regrowth of
202 microorganisms harboring ARGs that were non-functional due to regulation and codon usage
203 bias, which can result in simultaneous ARG presence and antibiotic sensitivity.[25, 26] SMX and
204 ERY are bacteriostatic antibiotics, halting growth but not necessarily lysing cells, and the only
205 two antibiotics present in significant concentrations in the effluent.[27] Growth of sensitive
206 ARG-harboring microorganisms may have been inhibited during the antibiotics phase,
207 resulting in low detection in the ARG profile. When the antibiotic selective pressure was
208 removed, the inhibited microorganisms may have increased growth rate, resulting in the
209 observed increase in ARG abundances.

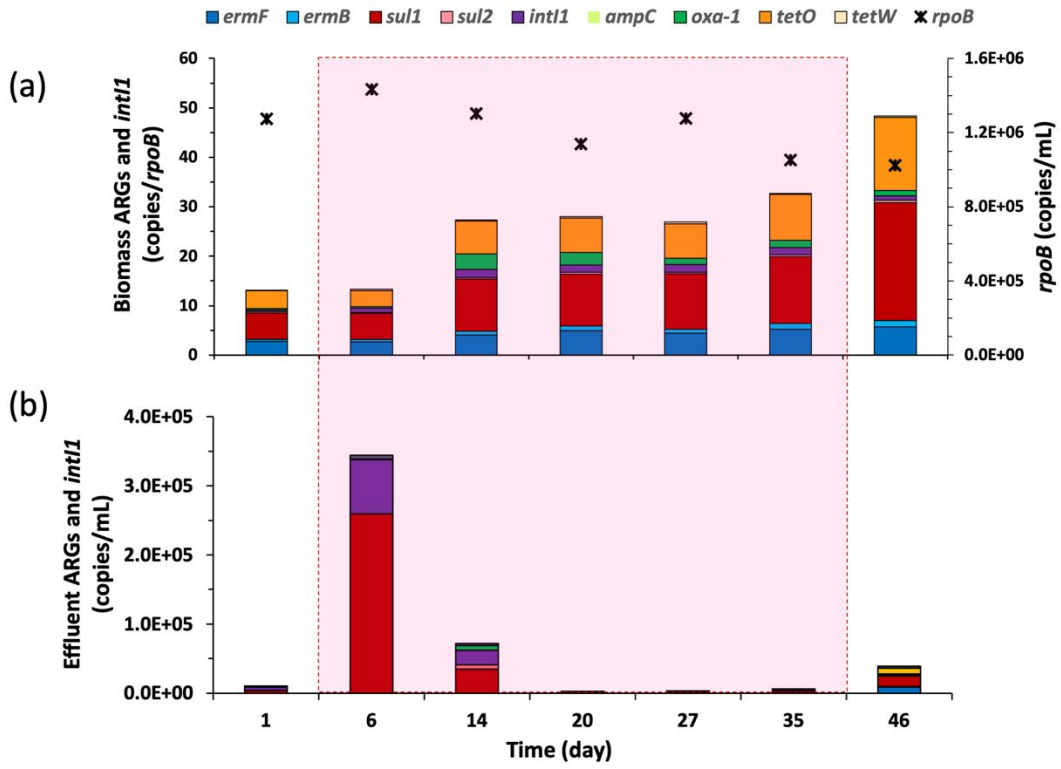



**Fig. 1.** Abundance of targeted genes in the (a) biomass (copies/rpoB) and (b) effluent (copies/mL) of the AnMBR throughout the experimental period. The x-axis represents the days after steady performance of the AnMBR was reached. Day 1 represents pre-antibiotics period, days 6, 14, 20, 27 and 35 represent antibiotics loading period (area bordered by red dashed line), and day 46 represents post-antibiotics period. The markers in (a) represent abundance of *rpoB* (copies/mL; secondary y-axis).

*sul1*, *tetO*, and *ermF* were the most dominant ARGs in the biomass profile, each respectively accounting for 41.3 ± 3.9%, 26.6 ± 2.4% and 16.7 ± 3.1% of targeted ARGs. Except for *sul1*, which was also the most abundant targeted ARG in the AnMBR effluent profiles (accounting for 53.6 ± 12.5%), the dominant ARGs of the biomass (*tetO* and *ermF*) accounted for less than 1% of total targeted genes in the effluent (except for day 46, as described above). Alternatively, *intI1* was the second most abundant gene in the effluent profile, accounting for 31.0 ± 14.6% of targeted ARGs. Abundances of targeted ARGs throughout the experimental

period in the biomass and effluent of the AnMBR are provided in ESI Table S4 and Table S5, respectively.

### 3.2.1. ARG abundance in AnMBR biomass increased throughout operation

After antibiotics addition on day 5, the abundance of class 1 integrons increased significantly in the biomass profile. However, no significant changes were observed in ARG abundances (Fig. 1a). Class 1 integrons are associated with HGT due to their presence on mobile genetic elements (MGEs) such as plasmids and transposons.[28] Thus, the significant increase in the biomass abundance of *intI1*, one day after antibiotics addition, could indicate a considerable rise in the presence of MGEs (and HGT). This rise may correspond to an increase in plasmid-based resistance within the biomass microbial community. This, along with an increase in the abundance of resistant microorganisms due to the antibiotic selective pressure, could have subsequently resulted in the significantly higher total biomass ARG abundances observed after day 6. Specifically, a marked increase in total ARG abundances from 13.3 ± 1.1 on day 6 to 27.3 ± 1.7 copies/*rpoB* on day 14 was observed. Biomass ARGs that increased on day 14 were *ermF*, *ermB*, *sul1*, *sul2*, *oxa-1*, and *tetO*. Increases in the abundance of the aforementioned ARGs under selective pressure of high antibiotics has been previously reported elsewhere.[8, 11] After the initial increase, abundance of quantified biomass ARGs remained approximately constant at 28.7 ± 2.6 copies/*rpoB* during the antibiotics loading period. However, biomass ARGs further increased significantly to 48.3 ± 3.4 copies/*rpoB* ten days after ceasing addition of antibiotics (day 46). It should be noted that with one sample for the post-antibiotics period, it is not possible to conclusively evaluate the response of the ARG profiles to removing the antibiotics selective pressure. Ultimately, both the initiation of antibiotics addition and its subsequent cessation at the final stage of the experiment likely corresponded with marked increases in total biomass ARG levels.

### 3.2.2. AnMBR effluent ARG abundance spiked upon initial antibiotics exposure

Quantified effluent ARG abundances increased approximately 34-fold one day after antibiotics addition, primarily due to increases in *sul1* gene abundance, along with the class 1 integrons-associated *intI1* (Fig. 1B). Since, these two genes (*sul1* and *intI1*) are commonly co-located on conjugative plasmids, the cause of this phenomenon may relate to the fact that plasmid/extracellular ARGs contribute significantly to the effluent ARG profiles in membrane-based reactors. To explain this drastic increase in *sul1* and class 1 integrons, it can be inferred that antibiotics addition induced a spike in the rate of HGT in the biomass that led to a temporary increase in biomass extracellular plasmid DNA. This could have manifested as a sharp increase in effluent extracellular plasmid DNA and, consequently, a significant rise in abundance of *intI1* and *sul1* genes in the effluent ARG profiles. We previously observed an increase in *sul1* and *intI1* abundance in AnMBR effluent during individual addition of antibiotics (SMX, ERY and AMP).[8] Further, it has been reported that antibiotic mixtures, as opposed to individual antibiotics, result in more pronounced changes in ARG abundance.[11] Therefore, the drastic increase in *sul1* and *intI1* abundance after simultaneous addition of SMX, ERY, and AMP at a relatively high concentration of 250 μg/L is not unreasonable. Another possible explanation for this drastic increase might be the considerable release of MGEs due to the lysis of bacterial cells caused by AMP, which is bactericidal. Eight days after the sharp spike, total abundance of effluent ARGs decreased by 5-fold, and then decreased another 42-fold on day 20. Effluent ARG profiles remained approximately constant thereafter for the rest of the antibiotics loading period. These reductions could be due to microbial loss of MGEs such as plasmid DNA along with lower HGT rates after the initial antibiotics exposure. Another possible explanation for the spike and subsequent reduction in total effluent ARG abundances could be the development of the membrane fouling layer (biofilm) and

subsequent biofilm-based ARG removal.[8, 29] However, given that the TMP was consistent during the experimental period, it is possible that the influence of the biofilm on the effluent ARG profile was negligible.

Comparing the ARG profiles in biomass with those of the AnMBR effluent, it is noteworthy that both *tetO* and *ermF* genes (among the most abundant targeted ARGs in the biomass) were hardly detected in the effluent. This likely implies that *tetO* and *ermF* gene presence on extracellular MGEs was limited while bacteria harboring these ARGs were concurrently retained in the AnMBR by membrane separation. However, this was not the case for all genes. For instance, the significant increase in biomass abundance of the *oxa-1* gene on day 14 corresponded with a considerable increase in its abundance in the effluent ARG profile of the same day.

Following the same trend as the biomass ARG profile, effluent ARG abundances also increased after cessation of the addition of antibiotics. Overall, ARG abundances in both biomass and effluent were affected significantly by the addition of antibiotics to the influent. However, the mechanisms that dictated the increases in effluent ARG abundances at the stages of antibiotics addition and cessation, respectively, may have been vastly different from each other. Specifically, the ARG increases seen at the beginning of antibiotics addition (days 6 and 14) were driven by different gene combinations (*sul1* and *intl1*) than those that dominated the gene profile after antibiotics addition ceased on day 46 (predominantly *tetO*, *sul1*, and *ermF*). Interestingly, the effluent ARG profile of day 46 was also highly similar to that of the biomass throughout the experiment (Fig. 1), this observation being confirmed by an unweighted PCA performed on all of the ARG profiles (ESI Fig. S3). One possible explanation for this phenomenon is that the effluent ARG increases on day 6 were caused by elevated occurrence of HGT, while the increases on day 46 were the result of changes in the microbial

296 composition of the effluent after antibiotics cessation. Supporting this hypothesis, several
297 anaerobic biomass-associated microbial populations increased in their relative abundance in
298 the effluent line on day 46. These groups included *Syntrophomonas* and *Dechloromonas*
299 genera that showed strong correlation with biomass-dominating ARGs (including *ermF*, *ermB*
300 and *tetO*). Microbial dynamics and specific correlations are discussed in more detail in the
301 following sections.

302 **3.3. Microbial community analysis**

303 **3.3.1. Biomass relative abundances showed remarkable stability during antibiotics addition**

304 Similar to the ARG profiles, the microbial community structure in the biomass was
305 distinct from that of the effluent. The biomass community structure across the experimental
306 period was highly stable. This stability of the microbial community even after the addition of
307 multiple antibiotics at concentrations of 250 µg/L is further indication of the ability of AnMBRs
308 to sustain treatment of high antibiotic-containing wastewaters (e.g., hospital wastewaters).
309 NMDS and AMOVA were employed (Fig. 2a) to confirm similarity of the biomass microbial
310 community at the genus level throughout the experimental period. Results revealed that all
311 biomass community samples clustered closely together with no statistically significant
312 changes ($p = 0.081$). Based on the results of our previously published study,[8] it was speculated
313 that variations in biomass ARG profiles during the addition of antibiotics to the influent could
314 have been primarily due to changes in the microbial community. However, given the high-
315 level of stability among community relative abundances before, during, and after antibiotics
316 addition observed in the present work, it is likely that these antibiotic-influenced changes to
317 the biomass ARG profile are primarily due to HGT and not microbial community alteration.
318 Occurrence of HGT in anaerobic digesters has been reported previously.[12, 30]

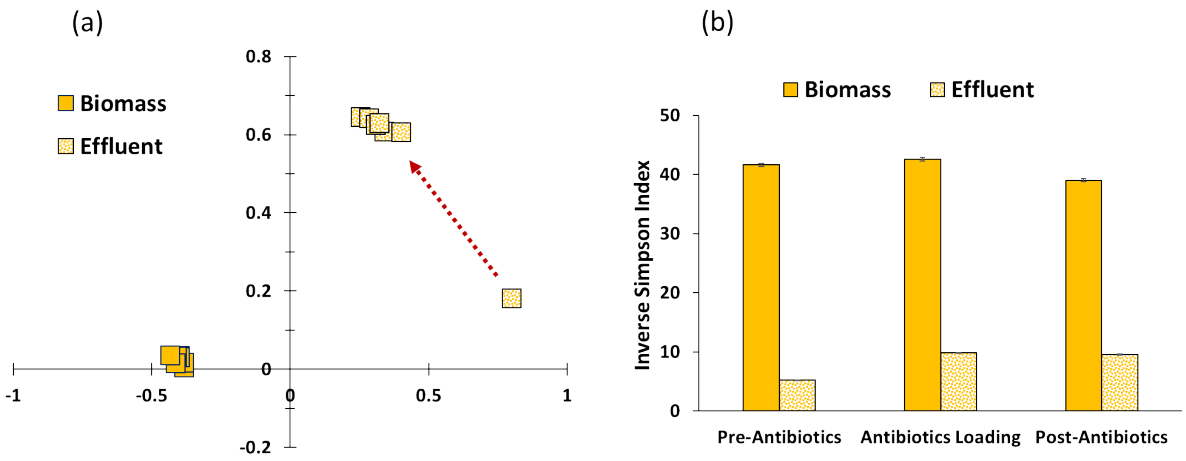



**Fig. 2.** (a) Non-metric multidimensional scaling (NMDS) and (b) Inverse Simpson index for the biomass and effluent of the AnMBR throughout the experimental period. The red arrow in the NMDS plot indicates the significant shift of the effluent samples after antibiotics addition. In the Inverse Simpson plot, the bars for antibiotic loading represent the average of the diversity index in n = 5 samples during the loading period (n = 1 for the pre- and post-antibiotics period). Error bars for the pre- and post-antibiotics period represent the standard deviation calculated by Mothur for each sample, and for the antibiotics loading period represents the standard deviations of the averages in n = 5.

Bacteroidetes (28.6 ± 1.96%), Chloroflexi (19.7 ± 1.2%), and Proteobacteria (13.2 ± 1.0%) were the most abundant phyla in the biomass. At the family level (Fig. 3a), the microbial community was comprised of 21.3 ± 2.1% unclassified Bacteroidetes and 11.0 ± 0.8% unclassified Chloroflexi. Only 25% of Bacteroidetes and 44% of Chloroflexi sequences were classified at the family level. Anaerolineaceae (8.59 ± 0.50%), Syntrophaceae (5.21 ± 0.33%), Ignavibacteriaceae (4.50 ± 0.27%), Methanoregulaceae (3.70 ± 0.20%), and Methanotrichaceae (2.77 ± 0.18%) were the most abundant classified families in the biomass community. Most genera associated with Anaerolineaceae are strict anaerobes that are commonly found in anaerobic treatment system for domestic wastewater[31] and anaerobic

digesters . The family Ignavibacteriaceae contains a single facultative anaerobic genus that is capable of utilizing aromatic compounds[32] and can also contribute to sulfide oxidation.[33]

Relative abundances of both methanogens and syntrophic fatty-acid oxidizing bacteria in the biomass remained stable throughout operation, averaging 8.68 ± 0.45% and 8.67 ± 0.55% relative abundance across the seven temporal samples (ESI Fig. S4). *Methanolinea* (3.73 ± 0.19%) and *Methanosaeta* (2.79 ± 0.18%) were the most abundant methanogens, indicating relatively comparable contribution of hydrogenotrophic (*Methanolinea*) and acetoclastic (*Methanosaeta*) methanogenesis. The most abundant syntrophs were unclassified Syntrophaceae and unclassified Syntrophorhabdaceae, with relative abundances of 3.96 ± 0.35% and 2.30 ± 0.20%, respectively. The family Syntrophaceae can oxidize long chain fatty acids to produce acetate and hydrogen,[34] however, the family Syntrophorhabdaceae mainly oxidizes aromatic compounds, such as benzoate.[35] Based on AMOVA analysis, antibiotics addition did not significantly affect the relative abundance of methanogens ($p = 0.108$) or their syntrophic counterparts ($p = 0.095$), indicating the robustness of these keystone microbial populations regardless of influent antibiotics concentration.

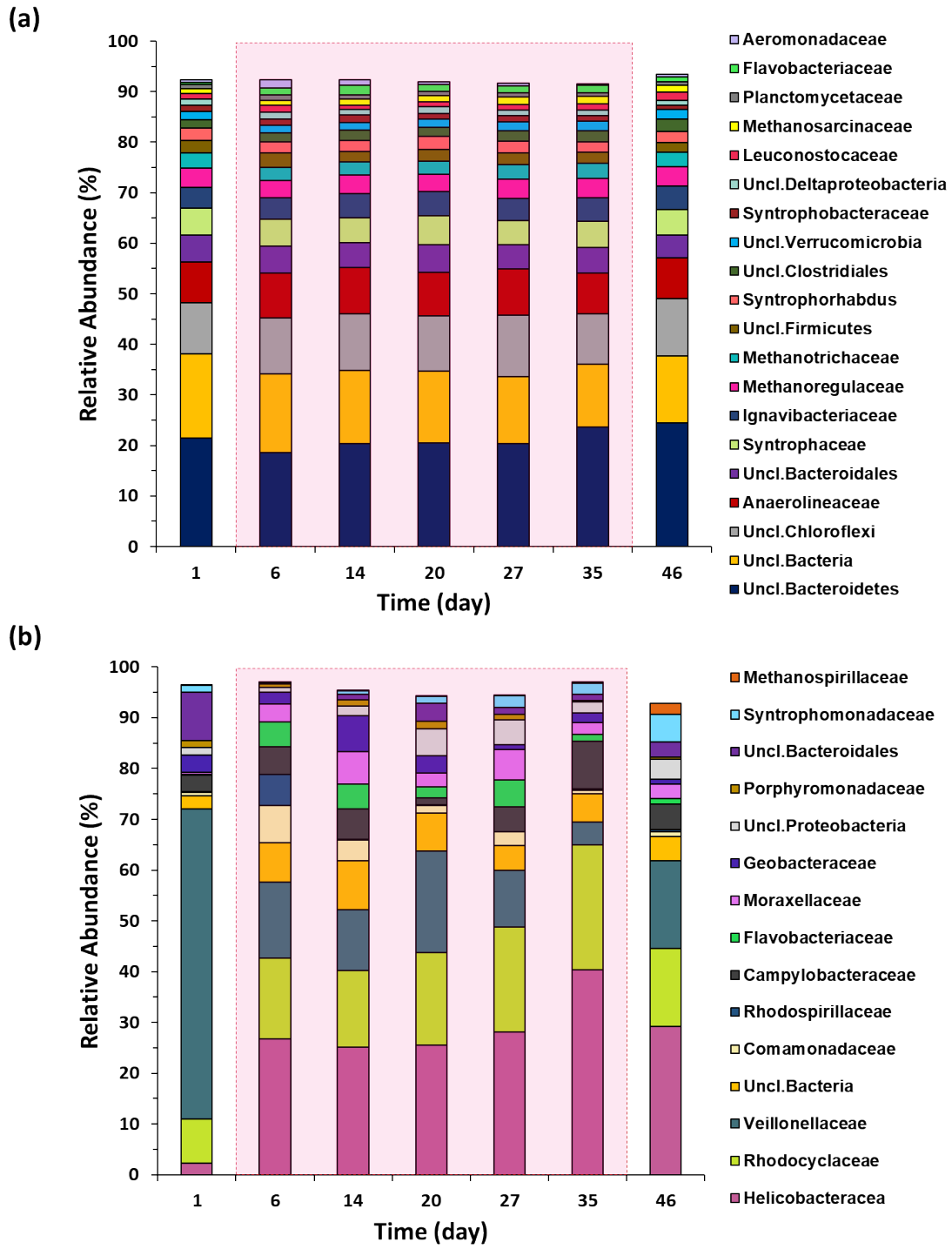



**Fig. 3.** Relative abundance of the (a) biomass and (b) effluent microbial community at the family level throughout the experimental period. Day 1 represents pre-antibiotics period, days 6, 14, 20, 27 and 35 represent antibiotics loading period (area bordered by red dashed line), and day 46 represents post-antibiotics period.

### 3.3.2. Dominant effluent microbial groups were affected significantly by antibiotics exposure

Effluent microbial communities were distinct from the biomass, as was clearly elucidated in the NMDS plot and AMOVA analysis ($p = 0.001$) (Fig. 2a). This is not surprising, owing to the fact that the 0.1 μm membranes used in this AnMBR system likely exclude passage of nearly all microorganisms present in the biomass. However, the addition of antibiotics to the influent did appear to significantly impact the effluent microbial community structure (which was not the case for AnMBR biomass). Since transmembrane pressure was consistent during the operational period at 19.3 ± 2.6 kPa, these changes in effluent community structure were likely not due to membrane fouling. Firmicutes (63.1%), Proteobacteria (22.5%), and Bacteroidetes (11.2%) were the most abundant phyla in the pre-antibiotics periods, whereas the dominant phyla in the effluent during antibiotics addition and in the post-antibiotics period were Proteobacteria (69.4 ± 7.7%), Firmicutes (15.7 ± 6.0%), and Bacteroidetes (6.47 ± 2.02%). Since these effluent changes during antibiotics addition did not concurrently change the biomass microbial community structure, further analysis of microbial diversity was performed to compare the biomass and effluent communities. Results revealed that the Inverse Simpson index of biomass sample in the pre-antibiotics period was 8 times higher than the effluent (Fig. 2b). This higher observed diversity indicates more evenness and richness among the biomass microbial community structure as compared to the effluent. The lower diversity of the effluent microbial community may have made this community more susceptible to inhibition, resulting in the high variability observed upon antibiotics addition.

At the family level (Fig. 3b), Veillonellaceae (61.07%) was the most abundant population in the pre-antibiotics period. A significant decrease to 13.3 ± 5.5% relative

abundance during the antibiotics loading and post-antibiotics periods suggests that members of this family are highly susceptible to inhibition from one or more of the introduced antibiotics. In contrast, the relative abundance of Helicobacteraceae, a sulfur-oxidizing family, significantly increased after addition of antibiotics from 2.24% in the pre-antibiotics period to 29.2 ± 5.7% during the antibiotics loading and post-antibiotics periods. Selection of the Helicobacteraceae family after antibiotics addition suggests a high likelihood of multi-drug resistance. Rhodocyclaceae, another prominent member of the effluent community, increased to 18.3 ± 3.8% after antibiotics addition. Within Rhodocyclaceae, *Zoogloea* is an aerobic genus that has been commonly found in activated sludge systems.[36] The enrichment of this genus in the effluent (ESI Fig. S5) might be an indication that aerobic bacteria likely increased their presence over time in the effluent tubes (which discharged to the open air). However, enrichment of *Zoogloea* in the effluent of anaerobic reactors has been reported frequently.[13, 37] Further, several effluent genera have been routinely isolated from biofilm samples: *Novispirillum* (Rhodospirillaceae), *Arcobacter* (Campylobacteraceae), *Comamonas* (Comamonadaceae), and *Aquabacterium* (Comamonadaceae).[38-41] These genera are mostly facultative anaerobes or capable of growth under anaerobic conditions. Therefore, their presence in the effluent may be due to seeding and regrowth as a result of membrane permeation by even a relatively small number of bacteria.

**3.4. Correlations between ARGs and microbial community structure indicate a potential for HGT**

To further investigate associations of ARGs with microbial communities in the AnMBR, a Spearman's correlation analysis was performed between ARG abundances and relative abundance of operational taxonomic units (OTUs) for the biomass and effluent samples (ESI Table S6). We elected to use an out-based approach for correlation analysis due to the large

406 proportion of unclassified sequences at the genus level. Network analysis was used to
407 illustrate statistically significant strongly positive correlations (Fig. 4) due to the implications
408 that positive ARG-microbial correlations have on ARG association with specific bacterial
409 groups.[42] The analysis revealed numerous strong positive correlations between certain ARGs
410 and microbial groups (OTUs) in the biomass. Although statistically significant positive
411 correlation between a microbial group and a particular ARG cannot be considered as evidence
412 of that group carrying antibiotic resistance, it can indicate the OTUs that are potential host
413 bacteria for ARGs and MGEs.[43] Several of the OTUs in the AnMBR biomass showed strong
414 correlations with multiple ARGs. Therefore, these groups may have a greater likelihood of
415 serving as potential multi-resistant host bacteria. Based on this and Table S6, OTU6
416 (Bacteroidetes), OTU32 (Clostridiales), OTU35 (Verrucomicrobia), OTU84 (Firmicutes),
417 OTU105 (Anaerolineaceae), and OTU134 (Bacteroidetes) in the biomass microbial community
418 were identified as potential multi-resistant host bacteria for *ermF*, *ermB*, *sul1*, *sul2*, *ampC*,
419 and *tetO* genes. Occurrence of multi-drug resistant bacteria in anaerobic environments, such
420 as anaerobic digesters, has been reported previously via strong correlations between
421 microbial community structure and ARG abundance.[15, 44]

422 Effluent correlations bear strong implications to the bacterial types that are actually
423 entering the environment through effluent discharge and/or reuse, and therefore are of
424 particular interest. The correlation analysis resulted in the effluent microbial communities
425 being divided into five distinct groupings: OTUs with strong correlations to (1) *sul1* and *intI1*,
426 (2) *sul2* and *oxa-1*, (3) *ermF*, *ermB* and *tetO*, (4) *tetW*, and (5) genera with no correlations to
427 ARGs. Based on Table S5, Group 1 included OTU25 (Bacteria), OTU27 (*Arcobacter*), OTU66
428 (*Novispirillum*), OTU78 (*Comamonas*), OTU89 (*Flavobacterium*), OTU159 (*Acetobacteroides*),
429 OTU247 (Bacteria), OTU264 (*Rhodococcus*), OTU352 (*Caulobacter*), OTU441 (*Sulfuricurvum*),

OTU501 (Caulobacteraceae), OTU550 (*Chryseobacterium*), and OTU648 (*Bdellovibrio*). It is possible that these groups can serve as potential hosts for *sul1*-combining class 1 integrons, thus also being implicated in HGT. *sul1* and class 1 integrons have been previously found on the same gene cassettes in different *Arcobacter*[46] and *Comamonas*[47] species in wastewater and soil samples. Strong correlation of *Flavobacterium* with these two genes has also been reported in drinking water and soil samples.[48-50] Perhaps most importantly, 7 OTUs of Group 1 are known to harbor pathogenic species. The presence of class 1 integrons in other pathogenic bacterial strains, including *Escherichia coli*,[51, 52] *Pseudomonas aeruginosa*,[53] and *Salmonella* spp.,[54] is well established. Thus, the observed dominance of Group 1 by potentially pathogenic groups might be an indication of the transferability of such MGEs to a broader range of clinically significant bacterial strains. However, one of the limitations of the present study was the inability to classify microbial groups at the species-level for identification of pathogens.

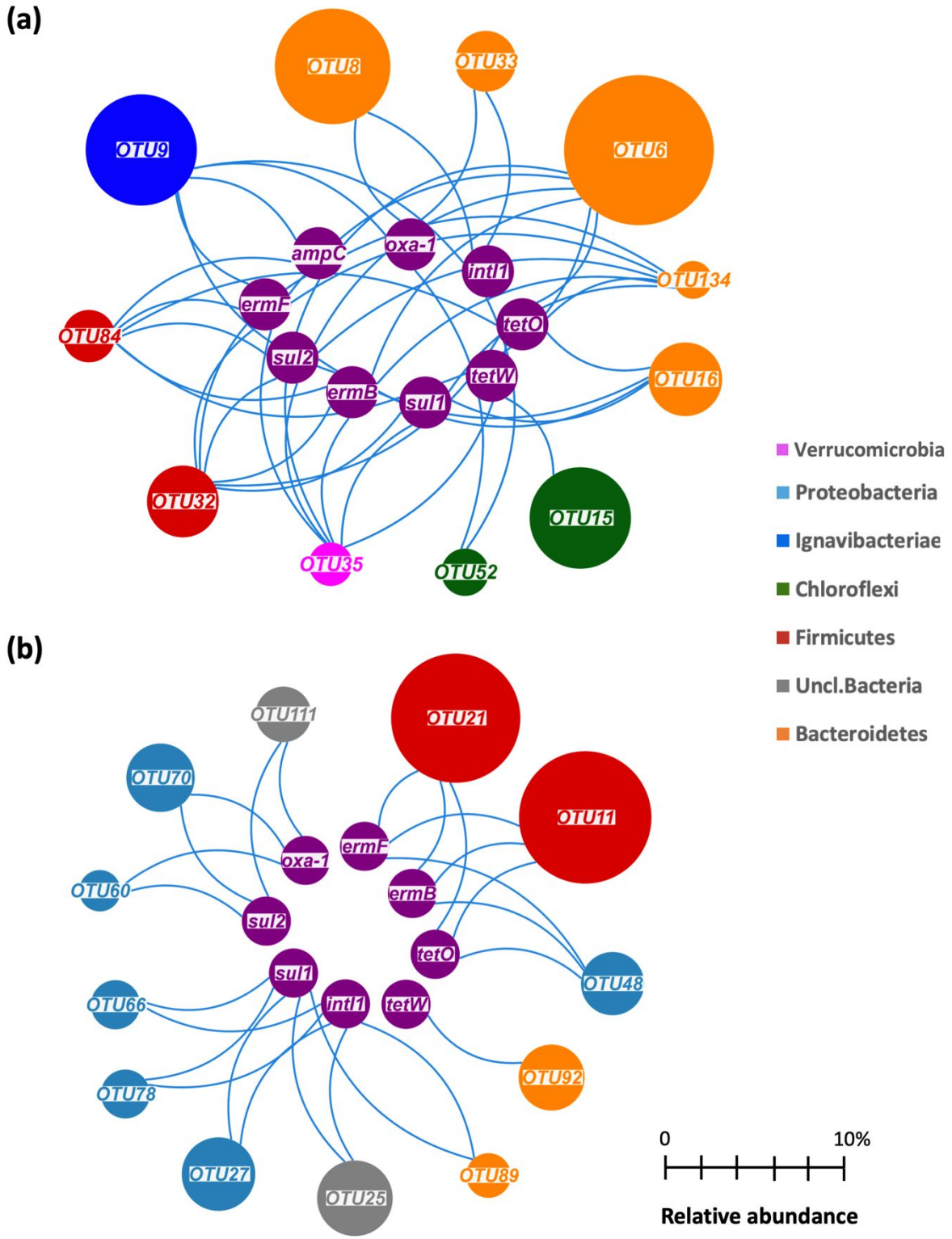
Group 2 (showing strong correlation to *sul2* and *oxa-1*) included OTU60 (Comamonadaceae), OTU70 (*Acinetobacter*), OTU111 (Bacteria), OTU163 (*Methylophilus*), OTU209 (*Desulfobulbus*), OTU287 (*Desulfovibrio*), OTU360 (Cryomorphaceae), OTU384 (Spirochaetaceae), and OTU499 (*Bosea*). Among group 2, some species of *Acinetobacter, Desulfovibrio,* Cryomorphaceae, Spirochaetaceae and *Bosea* might act as opportunistic pathogens. *Acinetobacter* spp. are among the six most important multi-drug resistant organisms in hospitals.[55] They are also frequently used as an antibiotic resistance indicator in water and wastewater.[56] The presence of *sul2*[57] and *oxa-1*[58] genes in different isolates of *Acinetobacter* spp. in wastewater samples has been reported previously. Although no putative association between the other members of group 2 and *oxa-1* have been reported in literature, in silico analysis of several Comamonadaceae species has revealed the presence

of open reading frames (ORFs) that correspond to OXA genes.[59] Further, strong correlation of *sul2* genes with Comamonadaceae in treated wastewater,[60] *Methylophilus* in a soil microbial fuel cell,[61] and *Desulfobulbus* during anaerobic digestion of cattle manure[62] have also been reported. Given that the co-location of *sul2* and *oxa-1* genes on plasmids has also been previously reported for multiple pathogenic strains,[63, 64] the observed correlations of group 2 with these genes might suggest their potential transferability to such species in the effluent.

Group 3 (strongly correlated with *ermF*, *ermB* and *tetO*) genera included OTU11 (*Anaerosinus*), OTU21 (*Propionispira*), OTU48 (*Dechloromonas*), OTU178 (Rhodocyclaceae), OTU184 (*Comamonas*), OTU299 (*Rhodopseudomonas*), and OTU389 (Bacteria). Strong correlations of *ermF* and *ermB* genes with a different tetracycline resistance gene (*tetX*) have previously been reported in multiple aquatic environments.[44, 65, 66] Further, the co-location of *ermB* and *tetO* genes in multiple *Enterococcus* spp. has been observed elsewhere.[67] Co-occurrence of *ermF* and *ermB* genes with *Comamonas*,[13] which is typically regarded as a pathogenic resistant bacteria,[68] has also been reported previously.

The forefront of the threat of antimicrobial resistance is specifically related to its ultimate transfer to pathogenic bacteria that can reach the environment. Thus, the dynamics of ARGs and MGEs in wastewater effluent microbial communities that are released to downstream water bodies and in reuse applications are of critical concern. This concern is further exacerbated when effluents contain significant levels of multiple pathogenic populations that can acquire these resistance elements through HGT. Correlation analysis of ARGs with microbial communities in the AnMBR effluent revealed some possible associations of potential pathogenic groups with at least one ARG. This is relevant, considering that several of these genera were correlated with Class 1 integrons, which implies a strong potential for HGT in the effluent environment. However, when putting these observations in the context

478 of AnMBR systems, specifically, it is important to note that previous studies have reported
479 significantly lower effluent abundances of pathogenic species in AnMBR effluents than in the
480 effluents of their aerobic MBR counterparts.[69] In the present study, a sharp decrease in
481 abundances of both ARGs and potentially pathogenic genera in the effluent were observed
482 after Day 6 of antibiotics addition and remained relatively low until the end of the
483 experimental period. This suggests that pathogenic and/or resistant bacteria in the effluent
484 were not sustained beyond the initial antibiotics exposure adjustment period. However, It is
485 noteworthy to state that since the community analysis of the present study was not classified
486 at the species level, no certain claims can be made about actual pathogenicity of identified
487 OTUs. Therefore, future research is needed to investigate AnMBR effluents with respect to
488 the association of antibiotic resistance with pathogenic species.




489

490 **Fig. 4.** Network analysis representing the positive correlations between ARGs (purple circles)

491 and microbial structure (OTUs) with ≥ 0.5% relative abundance in at least one sample in the

(a) biomass and (b) effluent of the AnMBR. A connection shows strong significant positive correlation ($p < 0.05$; and $\rho > 0.7$). The bubble size is indicative of relative abundance.

## 4. Conclusions

AnMBRs are an emerging biotechnology for mainstream wastewater treatment with the potential to enhance energy efficiency and effluent reuse, while also theoretically lessening the spread of antibiotic resistance to the environment. In this study, a bench-scale AnMBR was employed to treat domestic wastewater containing antibiotics and investigate the association of microbial communities with ARG profiles in both biomass and effluent of the AnMBR. The main conclusions of the experiment are as follows:

- Performance of the AnMBR regarding COD removal, biogas production, and methane yield was robust during the simultaneous addition of three antibiotics at 250 μg/L.
- ARG profiles and temporal trends in the biomass of the AnMBR were markedly distinct from those of the effluents.
- Effluent ARG abundance spiked upon initial antibiotics exposure, mostly due to the significant increase in *sul1* and class 1 integrons. It then gradually decreased by around 167-folds and remained constant during the rest of antibiotics loading period.
- Biomass microbial community structure was unaffected by antibiotics addition and was relatively uniform throughout the experimental period.
- Antibiotics addition significantly influenced effluent microbial community structure.
- Correlation analysis revealed the existence of potential multi-resistant host bacteria in the biomass, while also showing that the effluent microbial community contained distinct groups of bacteria with varied potential mechanisms of resistance.

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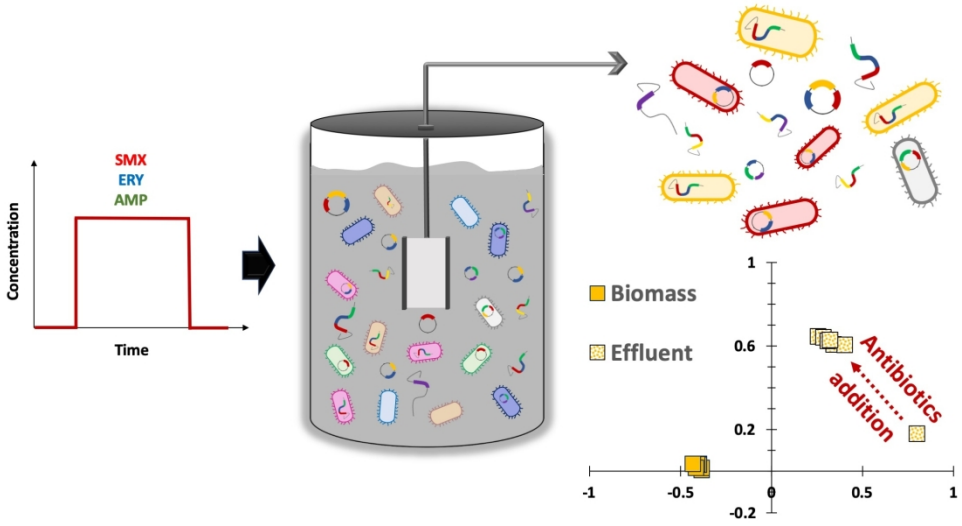


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