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Reducing the dissemination of ARGs and MGEs is critical for limiting human exposure to emerging health threats, particularly in water reuse. This study investigates ARG and MGE removal during co-treatment of domestic wastewater and livestock manure in an anaerobic membrane bioreactor (AnMBR). Results show an AnMBR can effectively treat complex wastes and limit the spread of ARGs in the environment.

Livestock manure improved antibiotic resistance gene removal during co-treatment of domestic wastewater in an anaerobic membrane bioreactor†

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Abstract

Anaerobic membrane bioreactors (AnMBRs) can manage complex combined waste streams, recover energy, and produce nutrient-rich effluents for irrigation. To advance AnMBRs for water reuse, the removal of antibiotic resistance genes (ARGs) during co-treatment of waste streams requires further attention. Here, an AnMBR was fed domestic wastewater with increasing amounts of cattle manure. The removal of target genes including nine ARGs and two mobile genetic elements (MGEs) was assessed. Manure addition was found to significantly improve the removal of target genes, with a removal efficiency of 99.95% during the stage with the greatest addition of manure. Further, the effluent contained more cell-free ARGs (cfARGs) than cell-associated ARGs (caARGs) when manure loading was greatest. This study is the first to evaluate ARG and MGE removal during co-treatment of domestic wastewater and livestock manure using AnMBRs, and also the first to differentially characterize the cfARGs/caARGs in an AnMBR effluent treating complex waste streams.

Keywords: Anaerobic membrane bioreactor, antibiotic resistance genes, wastewater, manure, cell-associated ARGs, cell-free ARGs

1. Introduction

Seventy percent of the world's freshwater supply is currently used for agricultural irrigation,[1] which is driving the adoption of nontraditional water sources to ensure the long-term supply of water for crop production. Treated wastewater represents a reliable source of water that can offset the rate of freshwater depletion while concurrently reducing nutrient discharges into freshwater systems and redirecting those nutrients to offset fertilizer requirements. This is especially relevant considering that traditional approaches to crop fertilizer delivery (i.e., application of manure) are coming under increasing scrutiny because of their greenhouse gas emissions, lack of energy valorization, and nutrient runoff issues.[2] Given that approximately 335 million tons of manure are produced each year in the United States.[3] the co-management of manure and wastewater is an attractive approach for advancing the use of alternative water sources for irrigation. Anaerobic membrane bioreactors (AnMBRs) are an emerging biotechnology that are ideally suited to co-manage manure and wastewater because they produce a high-quality, nutrient-rich effluent for reclaimed water irrigation while also recovering energy in the form of biogas.

One of the primary obstacles to reuse of treated wastewater for agricultural irrigation is the presence of emerging contaminants such as antibiotics, pathogens, antibiotic resistant bacteria (ARB), and antibiotic resistance genes (ARGs). Antibiotic resistance has been deemed one of the most significant human health challenges of the 21st century. Wastewater treatment plants represent a hotspot for the dissemination of ARGs in the environment.[4] Even after treatment, the elevated concentrations of nutrients, salts, and

lytic phages in wastewater effluents can contribute to cell competency and transformation, which can further promote the uptake of ARGs by microbial communities in receiving environments.[5] Thus, irrigation using reclaimed wastewater may lead to the dissemination of antibiotic resistance elements.[6,7]

ARGs have also been widely detected in livestock waste.[8–10] Considering that manure management frequently involves land application of biosolids, the dissemination of ARGs in soil is also a major source of antibiotic resistance loading on the environment.[11] ARGs have been found in significantly higher abundances in soil that has received manure application as compared to un-manured soil.[12,13] ARG removal by conventional livestock waste management technologies (e.g., anaerobic digestion and composting) has been observed as variable and limited.[9,14,15] However, the impact of co-managing wastewater and manure on effluent ARG abundances in water for reuse for irrigation has not been investigated.

AnMBRs have been successfully applied for both low-strength domestic wastewaters and high-strength organic waste streams (e.g., food waste and manure), illustrating their versatility in managing organic waste streams.[16,17] Furthermore, AnMBR systems in or proximal to agricultural areas could be used in decentralized applications to increase energy recovery while also managing livestock waste by co-treating wastewater with animal manure. Co-management of domestic wastewater with higher strength waste streams, such as animal manure, increases the potential for net energy positive treatment using AnMBR.[18] In addition, AnMBRs may be uniquely suited to reduce antibiotic resistance proliferation during the co-treatment of wastewater and manure because

membrane systems, in general, have been shown to achieve better ARG removal than conventional activated sludge systems.[19] A lingering question related to ARG removal by AnMBRs, however, is the extent to which cell-free ARGs (cfARGs; i.e. extracellular ARGs that are present due to lysis of dead cells or the secretion of DNA from live cells) are capable of passing through the membranes of these systems.[20]

To advance the application of AnMBRs for the co-management of wastewater and manure for water reuse applications, we need a better understanding of their ability to remove ARGs and mobile genetic elements (MGEs) during treatment. The goal of this study was to (1) determine if there is an association between the amount of cattle manure added to domestic wastewater and the corresponding removal efficiencies of target genes in AnMBR, and (2) investigate how manure addition impacted the profile of ARGs and MGEs and their dominant form (i.e., cell-associated vs. cell-free) in AnMBR effluent. This is the first study to characterize ARG removal during AnMBR co-treatment of real wastewater and manure, and the first study to differentially characterize effluent cell-associated and cell-free ARGs and MGEs from an AnMBR treating real waste streams.

2. Methods

2.1 AnMBR set-up and monitoring

A bench-scale AnMBR with a liquid volume of 4.5 L (Chemglass Life Science, Vineland, NJ) was operated at ambient temperature (average 17.7 ± 0.39 °C). The pH of reactor mixed liquor was spot checked routinely and the observed values ranged from 7.2 to

7.6). The reactor was equipped with three submerged membrane housings that each contained a flat-sheet silicon carbide ultrafiltration membrane (Cembrane, Denmark) with 0.1 μm pore size and 0.015 m^2 effective surface area. Headspace biogas was recirculated via transversally mounted sparging tubes to limit membrane fouling, while operating at a sub-critical flux of 5.27 $L/m^2/h$ (LMH) which yielded a hydraulic retention time (HRT) of approximately 19 hrs. The AnMBR was inoculated with anaerobic digester sludge from the Joint Water Pollution Control Plant (Carson, CA). The detailed configuration of the AnMBR and operational parameters are shown in ESI† Section 1.1.1.

The operation of the AnMBR consisted of 5 stages: Baseline operation, Stage 1, Stage 2, Stage 3, and Stage 4. Domestic wastewater used in this study was collected from the City of West University Place WWTP (Houston, TX), a full-scale activated sludge WWTP that treats an average of 2 million gallons per day. The wastewater samples were collected periodically under dry weather conditions and at the same time of day to avoid diurnal variations. The wastewater was immediately transported to the lab and stored at 4 °C before feed preparation. In Stages 1 through 4, manure slurry was added to the domestic wastewater in increasing amounts. Manure from beef cattle was collected from McGregor Research Center (McGregor, TX) and then diluted with water to form a manure slurry with a target solids content of 3%. Table 1 provides detailed influent composition along with the corresponding organic loading rate (OLR) for each stage. Across the different stages of treatment, the OLR increased from 0.6 kg $COD/m^3/d$ in Baseline operation to 2.5 kg $COD/m^3/d$ in Stage 4 operation.

Performance indicators including chemical oxygen demand (COD), soluble COD (sCOD), mixed liquor total and volatile suspended solids (MLSS/MLVSS), pH, volatile fatty acids (VFAs; including acetic acid, formic acid, propionic acid, butyric acid, and valeric acid) and headspace biogas were monitored and described in ESI† Section 1.1.2. In addition, chemical cleaning using 0.5% sodium hypochlorite was performed at the end of each operational stage to avoid the potential impact of membrane fouling on the experimental results, and to maintain a consistent flux across all operational stages. In addition, effluent tubing was cleaned periodically to remove downstream tube wall biofilms that may have formed.

2.2 DNA extraction with internal standards

Cell-associated DNA (caDNA) and cell-free DNA (cfDNA) were separated in order to quantify cell-associated ARGs (caARGs) and cell-free ARGs (cfARGs), respectively. Internal standards were spiked into all samples prior to DNA extraction. caDNA internal standards were *E. coli* cells containing a modified engineered plasmid (ESI† Section 1.2). The plasmid, pReporter_8 (RRID: Addgene_60568),[21] is a low-copy plasmid that was previously modified by knocking out the gene encoding green fluorescence reporter (GFP) and inserted with the methyl-halide transferase (MHT) gene found in *Batis maritima*.[22] A 112 bp region on MHT gene (primers in ESI† Table S3) was selected as the target for qPCR to quantify the initial concentration of standard spiked into the samples ($\mathbf{C_o}$ in Equation 1) and the amount recovered in each corresponding sample ($\mathbf{C_s}$ in Equation 1). *E. coli* cells were harvested from overnight culture supplemented with 34 μg/mL chloramphenicol, mixed well and aliquoted into equal volumes for: (1) spiking in the

influent and effluent samples, and (2) conducting plasmid extraction on three of the aliquots to get the reference copy number through qPCR ($\mathbf{C_o}$ in Equation 1).

cfDNA internal standards were pUC19 plasmids containing a target insertion for qPCR quantification (ESI† Section 1.2). The insertion was a 183 bp fragment of the *ARHGAP11B* gene, a human-associated gene that is specific to the brain neocortex (Florio et al., 2015). Approximately 10^8 copies of the plasmid were spiked into each effluent sample prior to filtration. The initial concentration of spiked cfDNA internal standard was calculated using the concentration of the plasmid stock used measured by Qubit and the volume spiked. The initial concentration of spiked cfDNA (C_o in Equation 1) was quantified by performing qPCR on the plasmid stock. The concentration of recovered internal standard was quantified using qPCR to determine $\mathbf{C_s}$. caDNA and cfDNA standards did not amplify in non-spiked influent, manure, or effluent samples in 40-cycles of qPCR (data not shown).

Influent and effluent samples were passed through membrane filters (mixed cellulose ester, 0.22 μm pore size, Millipore Sigma, MA) and caDNA was defined as DNA extracted from biomass retained on the filters, whereas cfDNA was defined as DNA in the filtrate. Five influent (30 mL each) and five effluent (350 mL each) samples were collected during each operational stage. caDNA was quantified in influent and effluent samples, and cfDNA was quantified in effluent samples only because the influent contained significantly higher cell counts than the effluent indicated by the concentration of *rpoB* (50 to 10^4-fold higher concentrations in the influent versus effluent). Right before the sample filtration step, both caDNA and cfDNA internal standards were spiked into samples and mixed well.

Membrane filters with caDNA were stored in 50% ethanol at -20 °C until DNA extraction. DNA extraction was performed using FastDNA SPIN Kits for Soil (MP Biomedicals, CA). cfDNA was analyzed in effluent samples by successively collecting the filtrate from the 0.22 μm filtration step and concentrating the DNA in the filtrate using an adsorption-elution method as described by Wang et al.[23] cfDNA samples were stored at -20 °C until DNA extraction using FastDNA SPIN Kits for Soil (MP Biomedicals, CA). Total DNA was quantified in caDNA and cfDNA extracts using Qubit 3.0 with the dsDNA HS Assay Kit (Invitrogen, CA).

2.3 Quantification of ARGs and MGEs

Target genes for qPCR quantification included 9 ARGs (*sul1*, *sul2*, *tet*(O*)*, *tet*(W), *ermB*, *ermF*, *ampC*, *blaOXA-1* and *blaNDM1*), 2 MGEs (*intI1* and *tp614*) and *rpoB* (coding for β-subunit of RNA polymerase) used for normalizing ARGs and MGEs to calculate relative abundance. These ARGs were selected because they are frequently detected in wastewater. In addition, we specifically included *erm* genes because the manure used in this study was collected from beef cattle that were fed Tylosin, a macrolide-class antibiotic that may have resulted in selective pressure for *ermB* and *ermF*.[24,25] A class 1 integron gene (*intI1*) and a transposon gene (*tp614*) were also included because they have been found to be associated with the transfer of ARGs and to play an important role in the evolution and proliferation of multi-drug resistant bacteria.[26–28] The qPCR reaction was carried out in triplicate with each reaction containing 10.5 μL that included Forget-Me-Not EvaGreen qPCR mastermix (Biotium, CA), 50 nM ROX (Biotium, CA), 500 nM of forward and reverse primers, PCR grade H_2O, and DNA template. Primers and qPCR reaction

conditions are provided in ESI† Table S3 and S4. Ten-fold serial dilutions of cloned plasmids with each target gene were amplified in triplicate for each qPCR assay. The efficiencies of the real-time qPCR assays for the target ARGs and MGEs ranged from 89.1% to 103%. R^2 values were greater than 0.99 for all qPCR assays. The limit of quantification (LOQ) for the target genes ranged from 6 to 920 copies/reaction, which was equivalent to 19 to 3070 copies/mL for 30 mL influent samples and 2 to 263 copies/mL for 350 mL effluent samples. Quality control steps for qPCR are detailed in ESI† Section 1.3.

The concentration of target genes in each sample (gene copy number/mL) was calculated using the following equation (1):

$$\text{Gene concentration } \left(\frac{\text{copies}}{\text{mL}}\right) = \frac{C_s C_i}{V_s C_o} \quad (1)$$

Where $\mathbf{C_s}$ is the copy number of the target gene in the sample's DNA extract (copies) determined by qPCR, $\mathbf{V_s}$ is the volume of sample used (mL) to generate the DNA extract, $\mathbf{C_i}$ is the copy number of the internal standard (copies) determined by qPCR, and $\mathbf{C_o}$ is the copy number of the internal standard (copies) spiked into the sample prior to DNA extraction. Further information on internal standards of cell-associated genes and cell-free genes along with the calibration methods can be found in ESI† Section 1.2. The recoveries of caDNA and cfDNA are provided in ESI† Table S6.

2.4 Statistical methods

SciPy (https://www.scipy.org) was used for t-test and correlation analysis. The two-tailed unpaired t-test was used to identify significant differences between effluent target gene concentrations between stages (n=5 for cell-associated genes; n=5 for cell-free genes).

The log removal values (LRVs) reported in this study for each gene for each operational stage were calculated using the following equation: LRV = log10(influent gene concentration/ effluent gene concentration). The influent and effluent gene concentrations were calculated by taking the average of n=5 samples. The effluent gene concentrations were the sum of cell-associated and cell-free fractions. A t-test was performed to assess whether a given LRV was significantly different between operational stages. Before performing a t-test, the Kolmogorov–Smirnov and Shapiro–Wilk tests were used to ensure that the dataset followed a normal distribution (α=0.01). P values less than 0.01 were regarded as statistically significant. Pearson's correlation analysis was used for identifying correlations between any pair of two target genes in effluent samples over a 95% confidence interval. Pearson coefficient (r) was used to identify strength of correlations.

3. Results & Discussion

3.1 Percent COD removal was consistent across all operational stages

Percent COD removal was measured across all operational phases to assess the impact of manure addition on AnMBR performance and effluent water quality. During Baseline operation where the influent consisted of only domestic wastewater (influent COD = 431 ± 42 mg/L), the effluent COD was 54 ± 12 mg/L and the average COD removal was 87.6 ± 2.27%, which is consistent with the range of COD removals reported in similar systems at psychrophilic temperature.[29] Upon increasing the influent COD in subsequent operational stages from under 500 mg/L to above 1600 mg/L COD through the addition of

196 livestock manure, the COD removal rate remained relatively constant, ranging between
197 87.0% to 89.7% (ESI† Fig. 1). This suggests that AnMBRs have the capacity to provide
198 consistent COD removal during the co-management of domestic wastewater and livestock
199 manure. The results are also consistent with COD removal efficiencies reported in previous
200 studies investigating anaerobic treatments with other high-strength organic substrates at
201 similar temperatures under mono-digestion conditions.[30,31] As the addition of manure
202 resulted in an increase in effluent COD across the Stages 1 through 4 (ESI† Fig. S1), the
203 OLR and ratio of influent manure to wastewater would have to be taken into consideration
204 depending on the final application of the effluent. Other performance data including
205 methane production are provided in ESI† Fig. S1, solids concentrations in ESI† Table S1,
206 and VFA concentrations in ESI† Table S2.

207 **3.2 Wastewater contributed the majority of influent ARGs during manure co-**
208 **treatment**

209 Although manure contributed the majority of influent COD in Stages 1 through 4
210 (Fig. 1A, $p<0.001$), the majority of influent ARGs and MGEs across those same stages
211 remained dominated by the domestic wastewater (Stage 1 shown in Fig. 1B, $p<0.05$). The
212 most abundant target ARGs and MGEs in the wastewater were *intI1*, *sul1* and *sul2*, which
213 was consistent with previous studies.[32,33] While approximately 97% of the target ARGs and
214 MGEs in the influent was contributed by the wastewater fraction during Stage 1,
215 erythromycin ribosome methylation genes (*ermB* and *ermF*) that confer resistance to
216 macrolide antibiotics were mainly contributed by the manure fraction (Fig. 1B). This result
217 is consistent with the fact that the manure was collected from cattle that were fed Tylosin, a

macrolide antibiotic. Tylosin is a commonly used in-feed antibiotic in both cattle and swine livestock farms, and previous studies have observed high occurrences of *ermB* and *ermF* in livestock wastes.[24,25]. Interestingly, the multi-drug resistance gene, *blaNDM1* was below the limit of detection in the influent and effluent samples during Baseline operation when the AnMBR was treating solely domestic wastewater (number of samples = 5), but was detected at concentrations above 10^2 copies/mL when manure was added to the influent (Fig. 1B). No studies to our knowledge have specifically investigated the presence of the *blaNDM1* gene in livestock manure; however, one study detected two *blaNDM1*-postitive bacteria strains in the soil around animal farms.[34]

3.3 The overall removal efficiency of target ARGs and MGEs from domestic wastewater was comparable to or greater than reported removal efficiencies of conventional wastewater treatment

Influent (cell-associated) and effluent (cell-associated and cell-free) ARGs and MGEs were quantified across all operational stages to calculate removal efficiencies. The LRVs of the target ARGs and MGEs ranged between 0.20 to 4.13 during Baseline operation when the AnMBR was solely treating domestic wastewater, with significant differences across stages (discussed in Section 3.4) and genes (Fig. 2A). During Baseline operation, 87.4% (0.90 log) of target ARGs and MGEs were removed (Fig. 2B). This removal efficiency is comparable to a study of two full-scale WWTPs, which reported a 89.0% – 99.8% removal of target ARGs.[35] High LRVs of *tet* genes, *tet*(O) (2.41) and *tet*(W) (4.63) reported here are consistent with previous findings of *tet* gene log reductions in membrane bioreactors treating domestic wastewater.[33,36] LRVs of *sul* genes, *sul1* (1.43) and

240 *sul2* (1.28), were comparable to LRVs reported in previous studies on WWTPs, which
241 ranged from 1.2 - 2.7 logs.[33,37,38] In addition, we observed high removal efficiencies of *erm*
242 genes. The LRVs of *ermB* and *ermF* were 3.39 and 3.48, respectively, and they were both
243 ~1.0 log higher than the LRVs reported from conventional WWTPs.[37,39] Although *erm*
244 genes in influent increased across stages due to manure addition, the ultimate concentration
245 in effluent was still less than 100 copies/mL which is lower than typically seen in biological
246 effluent in conventional WWTPs or even in the final disinfected effluents.[26,37,40] All ARGs
247 and MGEs were successfully reduced during baseline operation at over 87% removal, with
248 the exception of *ampC*, which increased in effluent samples. The enrichment of *ampC* may
249 have been due to the growth of organisms harboring this gene in the bioreactor. Enrichment
250 of certain target ARGs in terms of their relative abundance (ARG copy number normalized
251 by *rpoB* copies) was also observed for *sul1* and *blaNDM1*, despite the fact that their
252 absolute abundance decreased during the AnMBR treatment. Other studies have observed
253 enrichment of different ARGs across biological treatment compartments in WWTPs in
254 terms of both absolute abundance[41,42] and in relative abundance.[33,43] The inconsistent
255 patterns of ARG removal/enrichment during wastewater treatment underscore the
256 challenges of predicting the fate of ARGs released to the environment and need to develop
257 a more mechanistic understanding of the factors that control ARG proliferation and
258 attenuation during treatment.

259 To our knowledge, there is only one study on the fate of ARGs in AnMBRs treating
260 real domestic wastewater.[36] In this study, a higher LRV of target ARGs (3.3 to 3.6 log) was
261 found in the AnMBR treating primary clarifier effluent, but the mechanisms behind such

high ARG removal efficiency were not well understood. This study, in combination with the limited number of previous studies on ARG removal during AnMBR treatment on real and synthetic wastewater,[36,44] suggests that AnMBRs are effective at removing a large suite of diverse ARGs present in domestic wastewater. This could be an important advantage over conventional wastewater treatment for improving microbial safety during agricultural reuse, especially considering that AnMBRs have also been shown to also surpass pathogen removal rates observed for full-scale aerobic MBRs.[45]

3.4 ARG and MGE removal efficiency increased with increased manure loading

This study is the first to examine the impact of co-treatment of domestic wastewater and manure on ARG removal. Results generally showed that the addition of manure was beneficial to overall target ARG and MGE removal rates, which was strongly supported by the consistent trend of decreasing target gene concentration in the effluent through Baseline operation to Stage 4 (Fig. 2C). Further, as the fraction of manure added to the influent wastewater was incrementally increased, the removal efficiency of overall target ARGs and MGEs also increased steadily from Baseline operation to Stage 4 (Fig. 2B). In addition, the overall target ARG and MGE removal rate was largely driven by the removal of *sul1*, *intI1* and *sul2* genes (ESI† Fig. S2). The LRVs of *intI1*, *sul1* and *ampC* consistently increased from Baseline operation to Stage 4 due to manure addition ($p<0.01$). In Stage 4, the LRV of all target ARGs and MGEs reached 3.31, which was mainly due to the highest LRV of the most abundant influent MGEs and ARGs, namely, *intI1* (4.77) and *sul1* (3.54) (Fig. 2A). The removal efficiency of target ARGs and MGEs observed in Stage 4 was higher than previously reported values from several WWTP studies (Jilu Wang et al., 2015; Y. Yang et

al., 2014; Wen et al., 2016). The LRVs of genes *blaOXA-1*, *ermB, ermF,* tet(O)*, tet*(W) *and tp614* were consistently high across all stages (Fig. 2A; ESI† Table S7). Further, it was interestingly observed that *ermB*, *ermF* and *tp614* were significantly more abundant in the influent manure fraction as compared to the influent wastewater fraction ($p<0.01$, Fig. 1B). The higher influent concentrations contributed by manure addition may have increased the AnMBR's potential to remove them when manure was added compared to Baseline operation(when only the wastewater was treated). A previous study found that manure was dominated by cell-associated DNA as opposed to cell-free DNA (Zhang et al., 2013). We observed that *ermB*, *ermF* and *tp614* were significantly more abundant in the manure than the wastewater fraction of the influent ($p<0.01$, Fig. 1B). Thus, their superior removal when manure was added to the AnMBR may have been because the cell-associated DNA was readily removed via filtration.

The overall target ARG and MGE concentration in the effluent decreased consistently with the addition of manure (Fig. 2C, $p<0.01$), and in Stage 4 effluent, this concentration was approximately 90% lower than that of Baseline operation when the AnMBR was treating domestic wastewater without manure. The removal efficiency of target ARGs and MGEs observed in Stage 4 was higher than the previously reported values from several WWTP studies[39,41,46] and of manure treatment approaches including advanced anaerobic digestion[14,47] and composting.[48] This result, combined with the fact that AnMBRs can recover energy in the form of biogas, underscore that AnMBR is a strong candidate for the co-management of wastewater and manure because they can potentially reduce the proliferation of ARGs in wastewater and animal waste. The results support the

306 application of AnMBRs in decentralized agricultural applications where multiple waste
307 streams must be managed and water and energy reuse could be harnessed.

308 The improvement in ARG removal with increasing manure loading may have
309 resulted from enhanced microbial activity caused by the increasing OLR. The increase in
310 microbial activity may have impacted ARG removal in several different ways. First,
311 manure addition could have resulted in a shift in the microbial community to fast-growers
312 that could quickly break down the organics in the substrate. This, in turn, may have selected
313 for microbes that harbor fewer ARGs since they can exert a metabolic burden (i.e. fitness
314 cost) that can result in slower growth rates.[49] Second, the enhanced biological activity
315 resulting from a greater input of nutrients to the system may have boosted growth generally
316 and resulted in greater turnover of cells and biodegradation of DNA that included ARGs.
317 We also observed a consistent shift in the effluent DNA from cell-associated to cell-free
318 from Baseline operation to Stage 4 (Fig. 3). This observation supports the assumption that
319 manure addition resulted in an increase in biological activity in the bioreactor as a previous
320 study by Nagler et al.[50] in which it was found that the ratio of cfDNA to total DNA content
321 was significantly positively associated with biological activity in anaerobic digesters.
322 Further, the methane COD conversion normalized by the feed COD (Table 1) consistently
323 increased from Baseline operation to Stage 4 ($p<0.01$), supporting the hypothesis that
324 manure addition resulted in increased biological activity.

325 **3.5 The effluent ARG and MGE reservoir shifted from cell-associated to cell-free with**
326 **increased manure loading**

The majority of effluent ARGs and MGEs were cell-associated during all operational stages except for Stage 4 (ESI† Fig. S4). In Stage 4, the cell-free fraction of target genes was significantly elevated as compared to the previous stages (t-test, $p<0.001$) and accounted for approximately 89% of the target ARGs and MGEs in the effluent. At the same time, the concentration of the cell-associated fraction of target ARGs and MGEs in effluent decreased consistently from Baseline operation to Stage 4 ($p<0.01$). These results indicated that cfDNA became the primary reservoir of target genes in the effluent when the manure loading to the system was the highest. The abundance of effluent cell-free ARGs and MGEs confirms that they should not be overlooked in wastewater effluents (or unquantified because of the DNA concentration protocol used) (Fig.4), as they can make up a substantial fraction of effluent ARGs under some conditions.[37] The vast majority of studies on ARGs in wastewater did not explicitly capture the cell-free fraction of ARGs, and thus may have significantly underestimated the risk of ARG propagation from effluents in receiving environments. Previous studies that distinguished between cell-associated and cell-free ARGs using PMA-based PCR,[51] magnetic bead extraction,[52] or NAAP-based extraction (same as applied in this study)[53] found that most ARGs in WWTP effluent following disinfection were in extracellular forms or within cells with compromised membranes, underscoring the importance of accounting for cfARGs.

In the cell-associated fraction of effluent target genes, the abundance of *intI1* and *sul1* decreased significantly with the addition of manure across all stages ($p < 0.05$) (Fig. 2A). The removal of *intI1* may have been due to the elimination of manure-associated aerobic hosts of integrons (e.g., *Actinomycetales* and *Bacilli*) during anaerobic treatment.[54]

Overall, the cell-associated fraction of target ARGs and MGEs in the effluent decreased steadily from Baseline operation to Stage 4 (ESI† Fig. S4). Indeed, LRVs of the cell-associated fraction of target ARGs and MGEs across all five stages ranged from 1.54 - 4.20 logs and were consistent with reported ARG removal efficiencies using membrane-based treatment technologies.[33,55,56] Given that ultrafiltration membranes (0.01 - 0.1 pore size) retain the vast majority of microbes in AnMBRs, it is likely that some of the caARGs in the effluent are due to microbial regrowth within post-membrane effluent lines. Considering the higher effluent nutrient concentrations during the stages with higher manure addition, it is also possible that the observed reduction of cell-associated target genes was due to effluent selection for microbial groups which are less likely to harbor ARGs and MGEs due to fitness cost. We also performed correlation analysis to identify significant associations between effluent ARGs that were observed in cell-associated and cell-free fractions and the results are discussed in the ESI† Section 2.3.

Results of a previous quantitative microbial risk assessment (QMRA) on AnMBR effluents indicate that AnMBR treatment would likely need to be paired with a downstream disinfection process in agricultural reuse applications.[45] Based on this, the form of effluent ARGs (i.e., cell-associated vs. cell-free) may also influence their inactivation rates during disinfection (here, we define inactivation as the destruction of the ARG such that is no longer functional). Specifically, a greater proportion of ARGs in the cfDNA fraction may improve ARG inactivation during disinfection. Cell-associated ARGs are more difficult to inactivate because the cell serves as a barrier between the disinfectant and DNA and can thus protect the DNA against damage. A recent study evaluated multiple disinfection

methods including free chlorine, monochloramine, chlorine dioxide, ozone, UV, and hydroxyl radicals, and reported that among all these disinfection processes, caARG inactivation always lagged behind cell inactivation.[57] This indicates that the removal of caARGs requires cell inactivation to occur first, whereas cfARGs may be inactivated directly during disinfection. In a few of the very limited amount of studies that distinguished between caARGs and cfARGs during disinfection processes, caARGs were found to be more difficult to remove than cfARGs during chlorination and UV disinfection.[57–59] Further, other studies observed that caARGs became cfARGs during the disinfection process, indicating some disinfection may not be sufficient to completely destroy caARGs.[52,53] In this study, the concentration of effluent caARGs decreased steadily with increased manure loading (ESI† Fig. S4). In Stage 4, there were 2.34×10^4 copies/mL of caARGs in the effluent, which was much lower than secondary effluent caARG concentrations reported by previous studies of conventional WWTPs. Thus, our results show that application of AnMBRs for the co-treatment of domestic wastewater and livestock manure could reduce the proliferation risk potential during reuse, as they generate an effluent with relatively low ARG concentrations where cfARGs (which are easier to inactivate than caARGs) make up a substantial fraction of the total effluent ARGs and MGEs assessed in this study.

4. Conclusions

Our results demonstrate that co-management of domestic wastewater and livestock manure using AnMBRs can both improve resource recovery and mitigate the spread of

antibiotic resistance in reclaimed water. The removal efficiency of total target genes significantly improved with the increased manure loading in the AnMBR co-treatment process and was greater than many conventional WWTP treatment processes. Increasing manure loading not only decreased total target gene abundance in the effluent but also made cfARGs the dominant form of effluent ARGs. cfARGs require uptake by competent cells to be functional and are easier to inactive during disinfection. Thus, the ability of AnMBR to reduce ARGs during co-treatment of wastewater and manure and generate an effluent with primarily cell-free ARGs (as opposed to cell-associated) may be advantageous in water reuse applications.

Conflicts of interest

There are no conflicts to declare.

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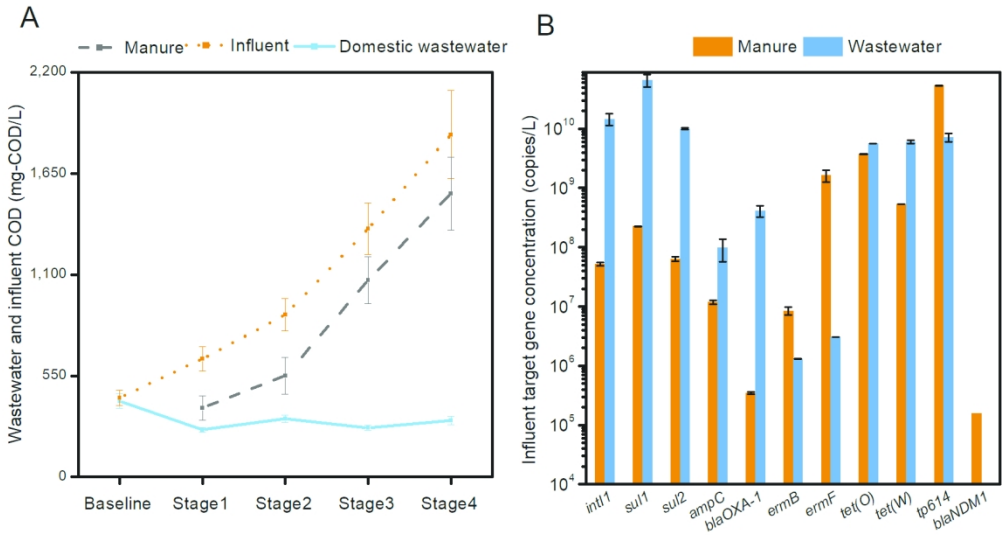

Fig. 1. AnMBR influent composition: (A) COD of wastewater and manure fractions of influent, and total influent COD across all operational stages. Error bars represent the standard deviation of biological replicates within each stage (n=5); (B) Concentrations of ARGs and MGEs during a Stage 1 loading (influent consisted of approximately 125 g of manure added to 20 L of domestic wastewater). Separate quantification of target genes in wastewater and manure was only performed during Stage 1. In all other stages, target genes were quantified in the influent after combining the wastewater and manure.

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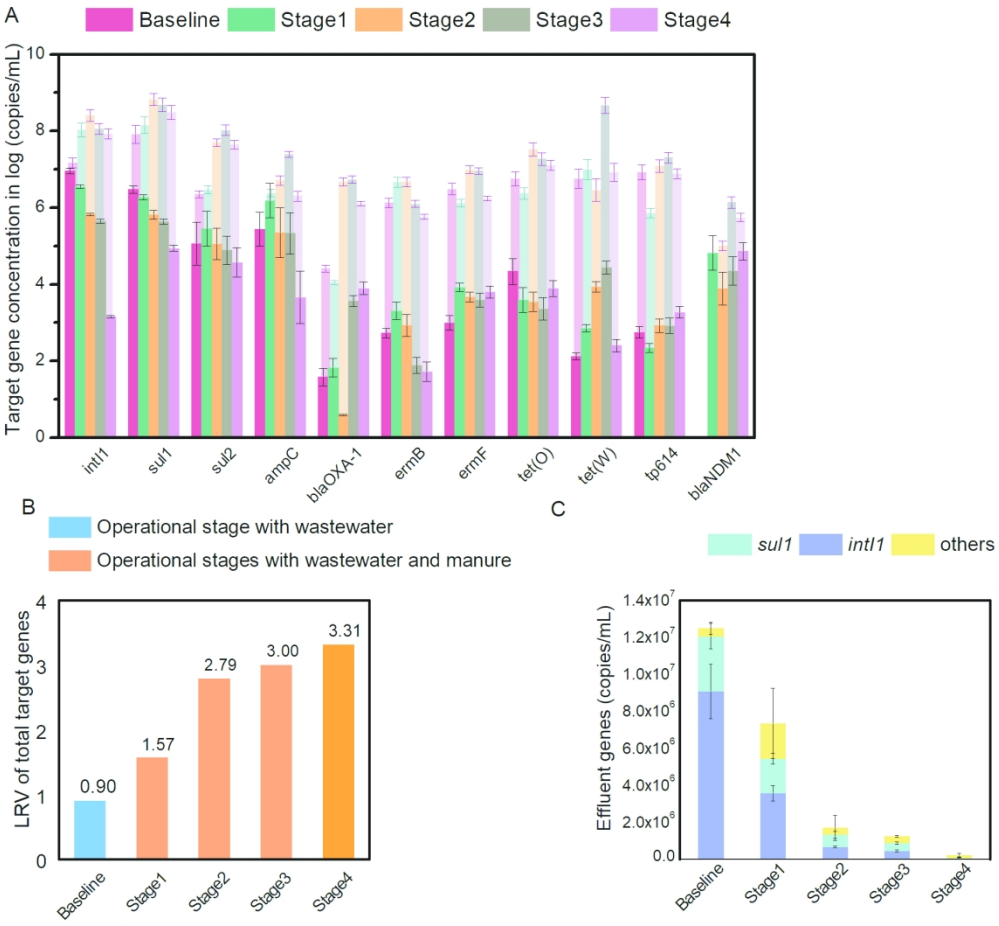

Fig. 2. ARG and MGE removal across operational stages: (A) Average influent (shaded bars) and effluent (solid bars) concentrations of target genes (n = 5 for influent and effluent samples); (B) Log removal values (LRVs) of total target genes across operational stages; (C) Effluent gene concentrations (sum of cell-associated and cell-free fractions; n=5). Error bars represent the standard deviations of gene concentrations within each operational stage.

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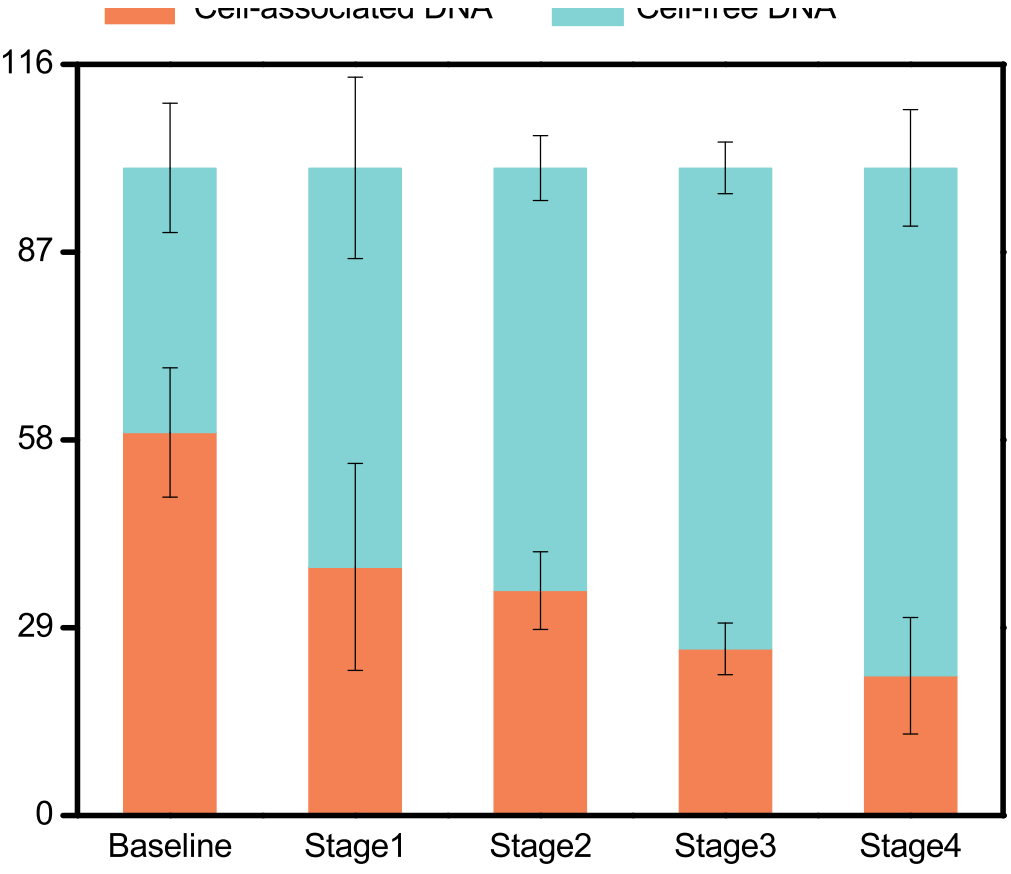
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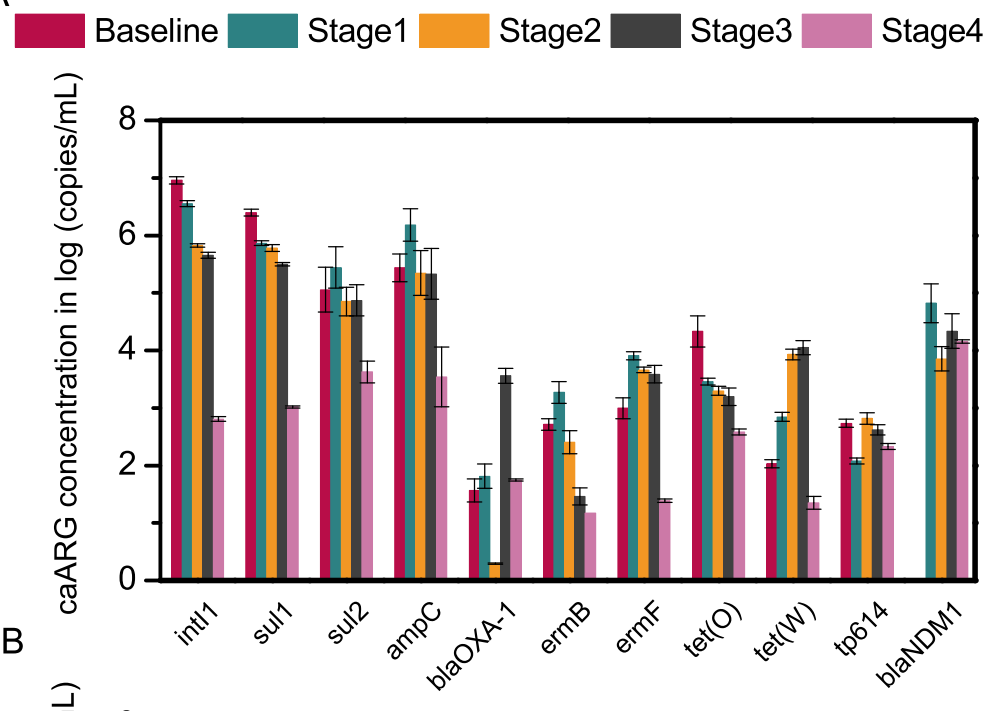
Stage2
Stage3
Stage4

A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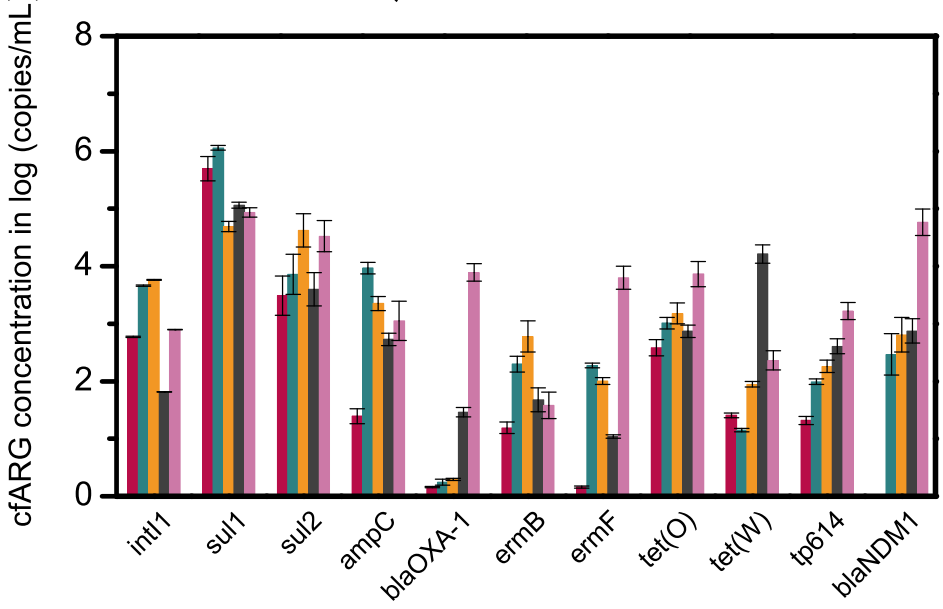
ermF
tet(O)
tet(W)
tp614
blaNDM1

B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intI1
sul1
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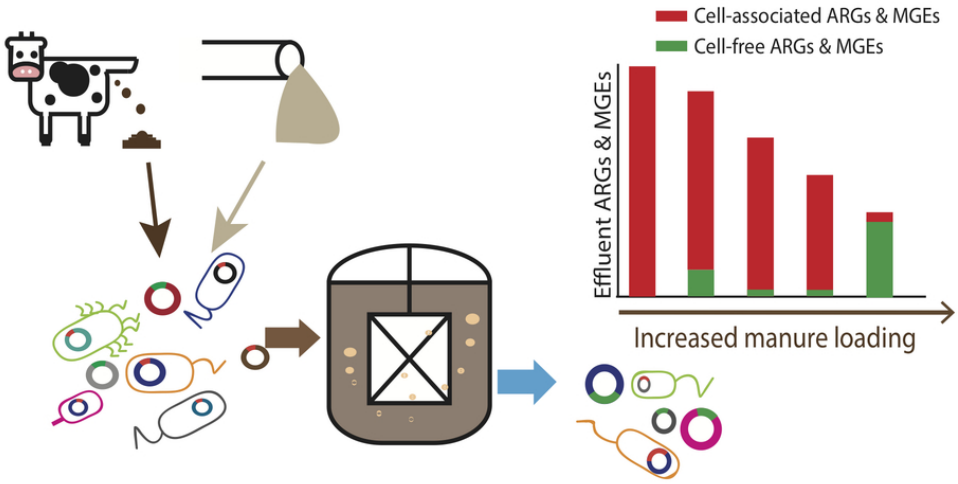
ampC
blaOXA-1
ermB
ermF
tet(O)
tet(W)
tp614
blaNDM1

Table of Contents Entry

First investigation of ARG and MGE removal in an anaerobic membrane bioreactor co-treating real wastewater and manure shows increased manure loading improves ARG and MGE removal.



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