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Molecular-based detection of potentially pathogenic bacteria in membrane bioreactor (MBR) systems treating municipal wastewater: a case study

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Running title: MBR-based removal of pathogenic species

Abstract Although MBR systems provide better removal of pathogens compared to conventional activated sludge processes, they do not achieve total log removal. The present study examines two MBR systems treating municipal wastewater, one a full-scale MBR plant and the other a lab-scale anaerobic MBR. High-throughput sequencing and digital PCR quantification were utilized to monitor the log removal values (LRVs) of associated pathogenic species, and their abundance in the MBR effluents. Results showed that specific removal rates vary widely regardless of the system employed. Each of the two MBR effluents' microbial communities contained genera associated with opportunistic pathogens (e.g., *Pseudomonas, Acinetobacter*) with a wide-range of log reduction values (< 2 to > 5.5). Digital PCR further confirmed that these bacterial groups included pathogenic species, in several instances at LRVs different than those for their respective genera. These results were used to evaluate the potential risks associated both with the reuse of the MBR effluents for irrigation purposes and with land application of the activated sludge from the full-scale MBR system.

Keywords pathogens; removal rates; aerobic; anaerobic; reuse; microbial regrowth

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Introduction

The issue of pathogen presence in treated wastewater effluents has gained attention recently due to an increased interest in reuse applications (Li et al. 2013; Zanetti et al. 2010). Previous studies have highlighted the advantages of aerobic MBR systems for the removal of microbial indicator bacteria (i.e. *E. coli*, total coliforms, fecal coliforms) from effluent discharges (Francy et al. 2012; Hai et al. 2014; Ottoson et al. 2006). Despite the high quality and low particulate effluents produced by MBR systems, it has been observed that 100% rejection of bacteria is not achievable by MBRs when operated with microfiltration (MF) membranes and that log removal rates (LRVs) vary based on the microbial indicator detected (Jong et al. 2010; Trinh et al. 2012; van den Akker et al. 2014). This variability in microbial removal rates ($< 10^4$ to $> 10^6$ removal) poses an obstacle for reuse purposes, as it means chlorine disinfection remains necessary for post-MBR effluents. Chlorination substantially reduces microbial risk but toxic and carcinogenic disinfection byproducts formed by chlorination can have a deleterious effect on effluents being applied for reuse (Krasner et al. 2009; Richardson et al. 2007).

An additional issue associated with aerobic MBRs, and activated sludge processes in general, is that of sludge production and disposal. Despite land application of sewage sludge being widely used throughout the world, pathogen-associated health effects of this practice are still of significant concern (Lewis and Gattie 2002; Lowman et al. 2013). This is, in part, due to inadequate treatment of sewage sludge before land application or disposal, especially in developing and industrialized countries (Pérez-Elvira et al. 2006). For example, a recent study assessing wastewater treatment practices in China found that

the vast majority of sludge treatment processes consisted of only sludge thickening and mechanical dewatering (Jin et al. 2014).

Given the limitations of aerobic MBRs, anaerobic MBRs (AnMBRs) have been viewed as a potential alternative municipal wastewater treatment technology due to their low sludge production rates, low energy use, and nutrient rich effluents (Smith et al. 2012). However, due to the lack of full-scale systems in operation, research addressing the microbial removal efficiencies of AnMBRs has been limited (Ellouze et al. 2009; Wong et al. 2009). Despite the inherently different effluent water composition (i.e. nutrient content) produced from AnMBRs compared to aerobic MBR effluents, there have not yet been any studies examining how these differences would impact the bacterial communities released into the environment. More specifically, there is a need to understand if and how the pathogenic bacteria present in wastewater influents would persist through AnMBR systems into their effluents.

A wide range of pathogenic bacteria are known to be present in municipal wastewater (Cai et al. 2014; Cai and Zhang 2013; Ye and Zhang 2011). Given that significant variability has been observed in the removal rates of indicator bacteria by MBRs in previous studies (Zanetti et al. 2010), a systematic assessment based on comprehensive molecular-based detection is therefore needed to determine the removal efficacies of aerobic and anaerobic MBRs. In particular, the use of high detection sensitivity methods such as high-throughput sequencing and digital PCR would be useful in addressing the removal of pathogens by MBRs (Bian et al. 2015; Cai and Zhang 2013).

The purpose of the present study is to employ high-throughput and digital PCR approaches to examine the specific presence and removal of potentially pathogenic bacteria in municipal wastewater by a full-scale aerobic MBR plant and a lab-scale anaerobic MBR system. It was further intended to apply results obtained from the molecular-based detection of specific pathogenic bacteria to evaluate the risks associated with both the reuse of MBR effluents and the disposal/application of the aerobic MBR activated sludge using quantitative microbial risk assessment (QMRA).

Materials & methods

Full-scale aerobic MBR system description and sampling protocol

The aerobic MBR evaluated in this study was a full-scale wastewater treatment plant receiving 6700 m^3/d of raw wastewater. The full-scale aerobic MBR (AeMBR) system consisted of the following process units: (i) primary clarifier, (ii) anoxic and aerobic activated sludge tanks, (iii) submerged membrane tank, and (iv) holding tank (Figure 1A). Membranes employed were flat-sheet 0.4 μm MF membrane cartridges by Kubota Membrane (Kubota Corporation, Osaka, Japan). A detailed description of the system operating conditions is provided in Appendix S1. Sampling was conducted between March 2015 and January 2016. Samples were collected from the influent, activated sludge, and MBR effluent as indicated in Figure 1A. Influent samples were prepared by centrifuging 30 to 100 mL of influent at 9400 *g* for 10 min to obtain a biomass pellet while effluent samples were prepared by filtering 2 L through a 0.4 μm polycarbonate membrane to retain the biomass. Finally, activated sludge samples were obtained by mixing 0.2 mL of sludge with 0.8 mL of 1X PBS solution and centrifuging at 9400 *g* for 10 min.

Lab-scale anaerobic MBR system description and sampling protocol

The anaerobic MBR (AnMBR) used in this study was a mesophilic up-flow attached-growth (UA) 2-liter anaerobic reactor as described previously (Harb et al. 2015). The reactor was connected in external cross-flow configuration to a 0.3 μm polyvinylidene difluoride (PVDF) MF membrane (Figure 1B). The system was fed with the same municipal wastewater being treated by the full-scale MBR plant. A detailed description of the reactor operational conditions is provided in Appendix S1. Sampling was

conducted over a 9-month period from April 2015 to January of 2016. The AnMBR effluent was sampled by filtering 0.5 L through a 0.4 µm polycarbonate membrane to retain the biomass. The AnMBR was also sampled for suspended and attached biomass using protocols described previously (Harb et al. 2015).

Water quality and biogas measurements

Water quality was monitored for both the AeMBR and AnMBR influents and effluents by measuring COD, ammonia, nitrate and nitrite content. COD of influent and effluent samples was measured using either LCK 314 (15-150 mg/L) or LCK 514 COD (100-2000 mg/L) cuvette test vials depending on the concentration to be measured. NH_4^+-N, NO_2^--N, NO_3^--N concentrations were measured using Test 'N Tube high range ammonia kit, TNTplus 839, and TNTplus 835, respectively. All measurements were conducted based on protocols specified by the manufacturer (Hach-Lange, Manchester, UK). Biogas produced from the AnMBR was captured continuously in gas bags from the headspace of the reactor and measured for volume, CH_4, O_2, N_2, and H_2 as described previously (Harb et al. 2015).

DNA extraction and 16S rRNA gene-based next generation sequencing

Genomic DNA was extracted using the UltraClean Soil DNA Isolation Kit (MoBio Laboratories,Carlsbad, USA) with slight modifications to the protocol by adding lysozyme and achromopeptidase to the lysis buffer (Hong et al. 2011). Illumina MiSeq amplicon sequencing was performed to provide information on the total microbial community. Details of the primers, PCR protocol, and quality control are presented in Appendix S2. Purified amplicons were submitted to the KAUST Genomics Core lab for unidirectional sequencing on an Illumina MiSeq platform. Raw sequence reads were

filtered to remove those determined to be chimeras and those with lengths of < 300 nt. Filtered sequence reads were analyzed using RDP classifier and an operational taxonomic unit (OTU)-based protocol as described previously (Harb et al. 2015). All high-throughput sequencing files were deposited in the European Nucleotide Archive (ENA) under study accession number PRJEB14612.

Species-targeted digital PCR (dPCR)

dPCR was performed to determine the relative abundances of species associated with opportunistic pathogens in influent, effluent and sludge samples. dPCR was performed using primers targeting *Acinetobacter baumannii* (McConnell et al. 2012) (*ompA*), *Klebsiella pneumoniae* (Lee et al. 2006) (*phoE*), and *Pseudomonas aeruginosa* (Lee et al. 2006) (*regA*). Relative gene abundances were normalized per liter of sample. *rpoB* gene copy numbers were also quantified to estimate total bacterial cell counts on the basis of single-copy gene homogeneity in all bacterial species (Dahllöf et al. 2000). dPCR was performed using the Clarity digital PCR System with a 32-tube reader (JN Medsys, Singapore) based on manufacturer's instructions. A description of the primers, dPCR protocol, detection sensitivity, and thermal cycling programs used are presented in Appendix S3. Primer sequences and their associated target species are shown in Table S1.

Quantitative microbial risk assessment (QMRA)

To further evaluate the potential microbial risks arising from reuse of the effluents of both the AeMBR and AnMBR, QMRA was performed for the three pathogenic species previously detected by dPCR. Additionally, the disposal of dewatered activated sludge was evaluated by QMRA for microbial risk of human exposure for *A. baumannii* and *K. pneumoniae* due to their detection in the AeMBR activated sludge. Bacterial cell counts for each pathogenic species were estimated based on *ompA, phoE and regA* all being being single-copy genes (Fitch et al. 1993; Hedstrom et al. 1986; Martiny et al. 2006). Probability of transmission of the bacteria was calculated based on an assumed value of 2.0×10^{-6} (Gerba and Choi 2006). QMRA was performed based on the main induction route for agricultural workers being dermal exposure to liquid particulates during irrigation events and induction to individuals land applying and/or land filling dewatered activated sludge being through dermal contact with sludge and accidental ingestion of particulates. The individuals potentially exposed to the sludge include workers involved in land application/disposal and other persons possibly entering disposal sites (Harder et al. 2014). Exposure assessment parameters were obtained from the USEPA exposure factors handbook (USEPA 2011). The k constants used for opportunistic pathogens were 2.76×10^{-7} for *A. baumannii* (López-Rojas et al. 2011), 1.05×10^{-4} for *P. aeruginosa* (Hazlett et al. 1978), and 1.62×10^{-6} for *K. pneumoniae* (Domenico et al. 1982) as determined by their LD_{50} dose based on an exponential model.

Point risk estimates were calculated using the following equation:

$$\text{Point risk} = 1 - e^{(k * \text{exposed dose})}$$

Annual risk estimates were further calculated using the following:

Annual risk = $1 - (1 - \text{point risk})^{\text{number of exposure days per year}}$

Annual risk was evaluated based on an acceptable microbial risk of 1×10^{-4} (Smeets et al. 2009). QMRA description and calculations for exposure dosages, point risk and annual risk values are provided in detail as Appendix S4.

Results

AeMBR and AnMBR water quality measurements and performance

COD removal for the AeMBR system was greater than 93% for all samples (Table S3). NH_4^+-N was detected in influent at an average concentration of 12.0 ± 2.8 mg/L and was undetected in AeMBR effluent samples. Conversely, nitrate but not nitrite was detected consistently in effluent samples at an average concentration of 15.2 ± 2.7 mg/L NO_3^-N, implying full nitrification by the system.

The AnMBR showed COD removal rates of 95-98% throughout operation (Table S4). Ammonia was detected in influent wastewater at an average concentration of 252 ± 4 mg/L NH_4^+-N while neither nitrite nor nitrate were detected. AnMBR effluent contained an average concentration of 242 ± 5 mg/L NH_4^+-N and no nitrate or nitrite, showing no nitrogen conversion by the AnMBR. The biogas produced by the AnMBR contained 72-78% methane, resulting in an average methane production of 241 ± 12 mL CH_4 / g COD.

Estimation of total bacteria by *rpoB* gene quantification

Copy numbers of the *rpoB* gene were quantified by dPCR to estimate total bacterial cell counts. Influent municipal wastewater contained an average total bacterial cell count of $2.26 \times 10^8 \pm 1.21 \times 10^8$ cells/L (Figure 2A) while AeMBR activated sludge contained $1.63 \times 10^9 \pm 2.19 \times 10^8$ cells/g. AeMBR and AnMBR effluent total bacteria were estimated at $1.91 \times 10^4 \pm 2.68 \times 10^3$ and $1.79 \times 10^5 \pm 8.22 \times 10^4$ cells/L, respectively. Based on these values, the AeMBR approximate LRV for total bacterial cells was 4.07 while for the AnMBR the LRV was 3.10.

Microbial communities of municipal wastewater influent

Relative abundances of 16S rRNA-based microbial classifications and *rpoB* gene-based total bacterial quantifications were used to estimate the levels of pathogen-associated genera in the municipal wastewater influent. The results of this analysis showed that 13 different pathogen-associated genera were identified in one or more of the wastewater influent samples. The most consistently detected genera in the influent of the MBRs included *Acinetobacter*, *Aeromonas*, *Arcobacter*, *Dialister*, *Escherichia*, *Pseudomonas*, *Stenotrophomonas*, and *Streptococcus* with at least 7 of 9 samples showing positive detection (Table 1).

Microbial communities of effluents of AeMBR and AnMBR systems

Similarities of the microbial communities of influent samples and effluents from the full-scale AeMBR and lab-scale AnMBR reactors were calculated using Bray-Curtis similarities and represented in an mMDS plot (Figure 3). Clustering of samples showed that effluents of both MBR systems were significantly different from the influent samples as well as from each other.

All of the genera identified in the municipal wastewater were also detected in the effluent of the full-scale AeMBR at least once (Table 1). Estimated LRVs based on samples containing the associated genera varied from 2.71 to 5.57. Genera with the highest estimated removal rates were *Acinetobacter*, *Arcobacter*, *Aeromonas*, *Dialister*, and *Streptococcus*, all of which showed LRVs of above 5. Conversely, *Mycobacterium* and *Legionella* showed the lowest reduction rates, with LRVs of below 3.

Of the 13 pathogen-associated groups detected in the influent, 5 genera were observed in the effluent of the lab-scale AnMBR system while 8 were undetected (Table 1). 4 of these 5 genera were seen consistently in the effluent with at least 8 of 11 samples showing positive detection. The detected genera included *Acinetobacter*, *Aeromonas*, *Arcobacter*, *Pseudomonas* and *Stenotrophomonas*. Estimated LRVs for these groups were 2.48, 3.85, 2.92, 2.46, and 1.73, respectively.

Detection of pathogenic species in MBR systems using dPCR

Specific pathogenic bacterial species were targeted by dPCR to determine their relative abundances in the wastewater influent and both full-scale AeMBR and lab-scale AnMBR effluents. The AeMBR sludge was also targeted due to the potential risk associated with disposal of the activated sludge (> 600 kg produced daily at the local wastewater treatment facility) while AnMBR sludge was not tested as anaerobic systems generally require little to no sludge wastage (SRT > 350 d). Samples were quantified for *Acinetobacter baumannii* and *Pseudomonas aeruginosa* as their associated genera were found in both MBR effluents. Due to consistent detection of unclassified *Enterobacteriaceae* in effluent samples, *Klebsiella pneumoniae* was also targeted.

Primers targeting the *ompA* gene revealed that all wastewater influent (n = 8) and AeMBR activated sludge (n = 8) samples showed positive detection for *A. baumannii* at $2.46 \times 10^6 \pm 1.89 \times 10^6$ copies/L and $3.61 \times 10^5 \pm 2.49 \times 10^5$ copies/g, respectively (Figure 2B). The average for the AeMBR effluent samples that showed detection was $4.99 \times 10^3 \pm 1.15 \times 10^3$ copies/L (3 of 7). In the case of the AnMBR effluent, only 1 of 11 samples indicated the presence of *A. baumannii* at a concentration of 1.67×10^2 copies/L.

For the effluent samples that showed positive detection of *A. baumannii*, estimated LRVs of 2.69 and 4.17 were calculated for the AeMBR and AnMBR systems, respectively.

Results revealed that *P. aeruginosa* was also present in all influent samples at $5.46 \times 10^3 \pm 3.58 \times 10^3$ copies/L but was undetected in any AeMBR activated sludge samples (Figure 2C). Despite not being present in the activated sludge, 4 of 7 AeMBR effluent samples indicated the presence of *P. aeruginosa* at an average *regA* gene concentration of $6.17 \times 10^2 \pm 5.58 \times 10^2$ copies/L. 2 of the 11 AnMBR effluent samples also showed positive detection with an average concentration of 3.34×10^2. The LRV rates for the AeMBR and AnMBR effluent samples indicating *P. aeruginosa* presence were 0.95 and 1.21, respectively.

K. pneumoniae was similarly detected in all 8 influent samples at an average of $7.37 \times 10^5 \pm 4.72 \times 10^5$ copies/L (Figure 2D). This species was further identified in 5 of 8 AeMBR activated sludge samples at a *phoE* gene concentration of $9.69 \times 10^4 \pm 3.22 \times 10^4$ copies/g. Effluents of both the AeMBR and the AnMBR also showed positive detection for *K. pneumoniae* at $5.91 \times 10^2 \pm 6.36 \times 10^0$ (3 of 7) and $9.70 \times 10^1 \pm 2.00 \times 10^1$ (5 of 11), respectively. This resulted in LRVs of 3.10 and 3.88 for *K. pneumoniae* in the AeMBR and AnMBR systems, respectively.

Quantitative microbial risk assessment (QMRA)

QMRA was performed for *A. baumannii, P. aeruginosa,* and *K. pneumoniae* to determine exposure doses imposed by AeMBR and AnMBR effluents on agricultural workers during irrigation activities. Average exposure doses and annual risks were calculated

based on 95% confidence intervals and are presented in Table 2. Full calculation results including point risk estimates and upper and lower interval bounds are provided in Appendix S4. Exposure doses from irrigation with AeMBR effluent for *A. baumannii, P. aeruginosa,* and *K. pneumoniae* were determined to be 114, 18.8, and 13.5 cells/event, respectively. These point doses resulted in average annual risk estimates of 6.02×10^{-3}, 1.88×10^{1}, and 4.17×10^{-3}, respectively (Table 2). In the case of the AnMBR effluent, event exposure doses were lower than that in AeMBR effluent and calculated to be 0.81, 3.23, and 2.35 cells/event for *A. baumannii, P. aeruginosa,* and *K. pneumoniae*, respectively. This resulted in annual risk estimates of 4.29×10^{-5}, 6.30×10^{-2}, and 7.28×10^{-4}, respectively.

The potential risks associated with the disposal or land application of activated sludge produced by the AeMBR were also evaluated using QMRA for *A. baumannii* and *K. pneumoniae* based on their detection in activated sludge samples (Table 2). Exposure doses were calculated for both dermal exposure and accidental ingestion of sludge by workers or individuals present at disposal/land application sites and used to estimate the annual risks associated with each. Exposure doses from dermal contact during disposal of AeMBR dewatered activated sludge were calculated to be 377 and 63.2 cells/event for *A. baumannii* and *K. pneumoniae*, respectively. These exposure doses resulted in associated annual risk estimates of 4.98×10^{-3} and 4.89×10^{-3}, respectively. Likewise, accidental ingestion doses of dewatered sludge during disposal were calculated for *A. baumannii* and *K. pneumoniae* as 1990 and 334 cells/event, respectively, resulting in respective annual risk estimates of 2.60×10^{-2} and 2.55×10^{-2}.

Discussion

Both the full-scale AeMBR and lab-scale AnMBR exhibited stable performance throughout the duration of each system's operation as well as differences in their respective nitrogen conversion rates. Overall microbial community structures of the effluents of each reactor were significantly different from influent wastewater microbial communities as well as from each other (Figure 3), implying that reactor type and water quality parameters can significantly affect effluent microbial community dynamics. Nonetheless, a range of pathogen-associated bacterial genera were found in the effluents of both systems at varying removal rates based on average influent wastewater concentrations. These findings confirm those of previous studies which indicate that although MBRs provide higher microbial removal rates than conventional wastewater treatment systems, effluents still contain detectable levels of potentially harmful bacteria (Francy et al. 2012; Zhang and Farahbakhsh 2007).

Existing research on the bacterial removal capacities of MBRs using culture-based methods has indicated that overall LRVs of total coliforms, *Escherichia coli*, and *Enterococcus* are in the ranges of 5.5-6, 4.5-6, and 4.6-6.2, respectively (Marti et al. 2011; Ottoson et al. 2006; van den Akker et al. 2014; Zanetti et al. 2010). The AeMBR examined in this study showed removal rates that were in a similar range with a total bacterial LRV of 4.1 and at least 6 pathogen-associated genera with LRVs of ≥ 4.5 (Table 1). Nonetheless, all 13 of the pathogen-associated genera present in the influent were also detected in the full-scale AeMBR effluent. LRVs ranged from as low as 2.71 (*Legionella*) to as high as 5.57 (*Arcobacter*). System operating conditions and water quality

parameters can potentially contribute to these differences. For example, the observed increase in nitrate concentration between influent to effluent samples could have favored denitrifying groups such as *Pseudomonas* (Carlson and Ingraham 1983) and contributed to their relatively lower LRVs (< 3.5).

Pathogen-associated genera in post-AeMBR effluents are a source of risk that can be easily mitigated by subjecting the effluent to chlorine disinfection (Wisniewski 2007). However, given the potential risks associated with disinfection byproducts, there has been recent interest in evaluating whether MBR effluents can be directly reused for irrigation and other applications (Purnell et al. 2016). As a result, the risks arising from pathogenic bacteria detected by dPCR to workers potentially irrigating with the full-scale AeMBR effluent were evaluated using QMRA. Results of this analysis showed that potential annual risks associated with this activity were above 6.0×10^{-3} for *A. baumannii, P. aeruginosa,* and *K. pneumoniae*. These values were higher than the average acceptable annual risk of infection of 10^{-4} (Smeets et al. 2009). Similar to what has been previously determined for post-secondary treated effluent from a conventional wastewater treatment plant (Al-Jassim et al. 2015), the findings in this study suggest that despite passing through an MF membrane, post AeMBR effluent still requires disinfection prior to use for irrigation activities.

Of the three specific pathogenic species targeted by dPCR, both *A. baumannii* and *K. pneumoniae* were found to be in relatively high abundance in the AeMBR activated sludge (Figures 2B and 2D). Conversely, *P. aeruginosa*, although present in both influent

and effluent samples of the AeMBR, was not detected in the activated sludge. Previous studies have also found *K. pneumoniae*, but not *P. aeruginosa*, at high concentrations in activated sludge (Dudley et al. 1980; Ju et al. 2016). These findings reiterate the need for an accurate evaluation of pathogen presence in activated sludge due to the environmental risks associated with sludge disposal and land application regulations (McCall et al. 2015; Wéry et al. 2008). Furthermore, due to the wide range of sludge pretreatment practices employed worldwide (Pérez-Elvira et al. 2006), the risk of pathogen exposure during land application of untreated dewatered sludge remains of major concern. The present study evaluated the annual risk of infection by *A. baumannii* and *K. pneumoniae* present in the full-scale AeMBR activated sludge in a dewatered state during sludge disposal/land application practices using QMRA. Results indicated that average annual risk by both dermal exposure and accidental ingestion were above 4×10^{-3} for both pathogenic species detected in the AeMBR sludge (Table 2), implying a significant potential health risk for workers and individuals exposed to dewatered sludge during disposal and land application activities. These results highlight the need for proper treatment of activated sludge prior to disposal (e.g. by anaerobic digestion) or by employing alternative technologies capable of sludge production minimization.

One possible alternative approach is the use of AnMBRs for wastewater treatment due to their inherently low sludge production rates. A similar evaluation of LRVs to that which was conducted for the AeMBR was hence also performed for AnMBR effluent to determine if it would be suitable for direct reuse. A relatively smaller number of pathogen-associated genera were detected in the lab-scale AnMBR effluent compared to

those in the wastewater influent (5 of 13). The LRVs of those genera, however, ranged from 1.73 (*Stenotrophomonas*) to 3.85 (*Aeromonas*), implying that the operating conditions and effluent parameters of the anaerobic system enrich for specific bacteria while removing others. Of the 5 pathogen-associated genera identified in the AnMBR effluent, those with the lowest LRVs (*Acinetobacter*, *Pseudomonas*, and *Stenotrophomonas*) have been previously determined to include high ammonia assimilating species (Sasaki et al. 2005a; Sasaki et al. 2005b). These groups were likely enriched for by the AnMBR's limited nitrification capacity (Table S4). Furthermore, given that all of the genera found in the effluent except for *Acinetobacter* are known to be either strictly or facultative anaerobic, the system's anoxic conditions likely facilitated the survival of these bacteria. *Acinetobacter*, a strictly aerobic bacterium, has been known to exhibit rapid adaptability and survival in anaerobic conditions (Zafiri et al. 1999), which potentially allowed for its persistence through the AnMBR system while other aerobic genera were fully removed.

Despite showing LRVs at the genus and family level in the range of 2.48-2.87 (Table 1), pathogenic species associated with *Acinetobacter* and *Enterobacteriaceae* (as detected by dPCR) showed higher removal in the AnMBR (LRVs of 4.17 and 3.88, respectively). These findings imply that although the effluent of the AnMBR likely enriched for several pathogen-associated genera, the abundances of their respective pathogenic species could be significantly lower. QMRA analysis of the AnMBR effluent for irrigation activities revealed that the annual risk of infection by *A. baumannii* was below the annual acceptable limit (4.29×10^{-5}). Conversely, *K. pneumoniae* was slightly above that

threshold (7.28×10^{-4}), while the annual risk for infection by *P. aeruginosa* was determined to be more substantial (6.30×10^{-2}). These results imply that, compared to AeMBR effluent, chlorination may be less crucial for the AnMBR effluent. This is useful for reducing the formation of disinfectant byproducts without significantly compromising associated microbial risks. However, for selected microbial groups (e.g. *P. aeruginosa*), additional process optimization measures or better management practices would be necessary to minimize occupational hazards and public health concerns.

One limitation of the present study is that it cannot be considered a comparison of AeMBRs and AnMBRs for pathogen removal, but is rather a case study of two systems with vastly different operational parameters. Nonetheless, the conclusions drawn from this study regarding the lab-scale AnMBR and its removal capacity are relevant to evaluating it as an alternative municipal wastewater treatment system. The AnMBR was generally effective at removing the pathogenic species targeted by dPCR with only a small number of the total effluent samples showing positive detection and relatively higher LRVs for those samples (Figure 2). Furthermore, the ability of anaerobic reactors to convert municipal waste to energy instead of sludge indicates that AnMBRs may be advantageous in addressing the microbial-based problems associated with wastewater treatment and sludge disposal.

Conclusions

Specific removal rates of pathogens can vary significantly regardless of the system employed. All of the pathogen-associated genera detected in the influent were also identified in the full-scale AeMBR effluent with a wide range of LRVs. The AnMBR was generally effective at removing the pathogenic species targeted by dPCR with only a small number of effluent samples showing positive detection. Nonetheless, QMRA analysis showed that despite favorable removal rates, direct reuse of the MBR effluents could still pose a substantial risk to humans. Likewise, the activated sludge produced from the AeMBR plant introduces an additional risk arising from land application or disposal practices. These findings emphasize the necessity for a comprehensive understanding of pathogenic removal rates from influent, as well as pathogenic presence in sludge and effluents through molecular-based approaches.

Table 1. Average number of cells/L of genera associated with opportunistic pathogens. Calculated based on 16S rRNA-based high-throughput sequencing relative abundances as determined by RDP Classifier database analysis and average total bacterial cell counts as determined by *rpoB* gene copy numbers, assuming one gene copy per bacterial cell. Log reduction values (LRV) are shown for both the full-scale AeMBR and the lab-scale AnMBR. The numbers of samples showing positive detection are shown in parenthesis. ND indicates that the genus was not detected in any of the samples of that type and (-) indicates total removal.

Genera	**Influent Avg. (n = 9)**	**AeMBR Eff. Avg. (n = 8)**	**AnMBR Eff. Avg. (n = 11)**	**AeMBR LRV**	**AnMBR LRV**
Mycobacterium	1.2×10^4 (1/9)	1.9×10^1 (5/8)	ND	2.81	-
Treponema	3.3×10^4 (5/9)	1.6×10^0 (1/8)	ND	4.32	-
Arcobacter	1.0×10^7 (9/9)	2.7×10^1 (7/8)	1.2×10^4 (11/11)	5.57	2.92
Neisseria	3.4×10^4 (3/9)	1.1×10^0 (1/8)	ND	4.49	-
Acinetobacter *	1.4×10^7 (9/9)	1.1×10^2 (7/8)	4.7×10^4 (11/11)	5.13	2.48
Pseudomonas *	2.4×10^5 (7/9)	7.7×10^1 (8/8)	8.1×10^2 (8/11)	3.49	2.46
Legionella	1.0×10^4 (3/9)	2.0×10^1 (7/8)	ND	2.71	-
Unclassified *Enterobacteriaceae* *	1.3×10^6 (9/9)	4.4×10^1 (6/8)	1.8×10^3 (7/11)	4.48	2.87
Escherichia	9.8×10^4 (8/9)	3.4×10^1 (1/8)	ND	3.46	-
Stenotrophomonas	1.6×10^5 (8/9)	2.2×10^1 (6/8)	3.0×10^3 (10/11)	3.87	1.73
Aeromonas	1.6×10^6 (9/9)	8.3×10^0 (5/8)	2.3×10^2 (4/11)	5.30	3.85
Streptococcus	1.0×10^6 (9/9)	8.5×10^0 (4/8)	ND	5.07	-
Enterococcus	1.2×10^4 (1/9)	5.5×10^0 (2/8)	ND	3.34	-
Dialister	3.9×10^5 (9/9)	2.3×10^0 (1/8)	ND	5.23	-

Table 2. Average exposure dosage and annual risk of pathogenic species at a 95% confidence interval as determined by quantitative microbial risk assessment (QMRA) for irrigation exposure with influent and AeMBR and AnMBR effluents as well as AeMBR activated sludge dermal exposure and ingestion for land application/disposal activities.

Exposure dose and annual risk of species	Influent wastewater - exposure	AeMBR effluent - exposure	AnMBR effluent - exposure	AeMBR sludge - exposure	AeMBR sludge - ingestion
A. baumannii exp. dose	1.31×10^{5}	1.14×10^{2}	8.08×10^{-1}	3.77×10^{2}	1.99×10^{3}
A. baumannii annual risk	9.99×10^{-1}	6.02×10^{-3}	**4.29×10^{-5}**	4.98×10^{-3}	2.60×10^{-2}
P. aeruginosa exp. dose	2.91×10^{2}	1.88×10^{1}	3.23×10^{0}	-	-
P. aeruginosa annual risk	9.97×10^{-1}	3.15×10^{-1}	6.30×10^{-2}	-	-
K. pneumoniae exp. dose	3.92×10^{4}	1.35×10^{1}	2.35×10^{0}	6.32×10^{1}	3.34×10^{2}
K. pneumoniae annu. risk	1.00×10^{0}	4.17×10^{-3}	7.28×10^{-4}	4.89×10^{-3}	2.55×10^{-2}

Figure legend

Figure 1. Schematic diagrams of both MBR systems sampled in this study. **(A)** Schematic of full-scale AeMBR WWTP. Sampling points are indicated by orange dots and include (1) post clarification influent (2) aerobic activated sludge (3) MBR effluent. **(B)** Schematic of lab scale AnMBR. Sampling points are indicated by orange dots and include (1) post-clarification influent (2) anaerobic sludge (3) AnMBR effluent.

Figure 2. Gene abundances associated with **(A)** total bacteria (*rpoB*), **(B)** *Acinetobacter baumannii* (*ompA*), **(C)** *Pseudomonas aeruginosa* (*regA*), and **(D)** *Klebsiella pneumoniae* (*phoE*) expressed per liter of sample for wastewater influent, AeMBR activated sludge, AeMBR effluent, and AnMBR effluent. Numbers of samples for which each gene was detected out of total samples are shown in parentheses below each column. Asterisks (*) indicate that sample groups are significantly different from all other groups of the same gene type (unpaired *t*-test, $P \leq 0.05$).

Figure 3. Microbial community metric multidimensional scaling plot (mMDS) for the (A) influent wastewater used for both systems, (B) full-scale AeMBR effluent and (C) AnMBR Effluent. Black colored symbols represent the centroid of all samples of one type.

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Figure 1

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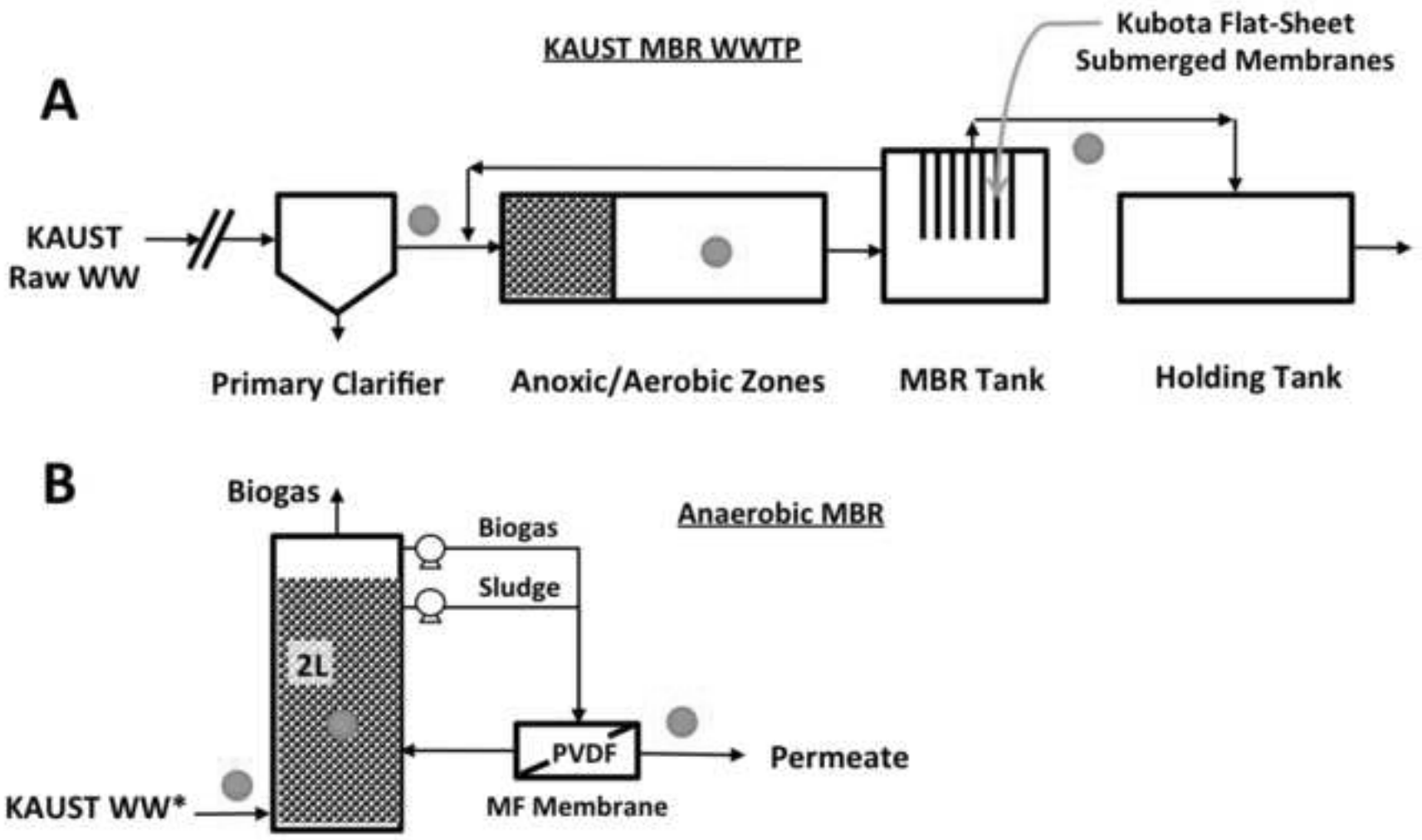

Figure 2

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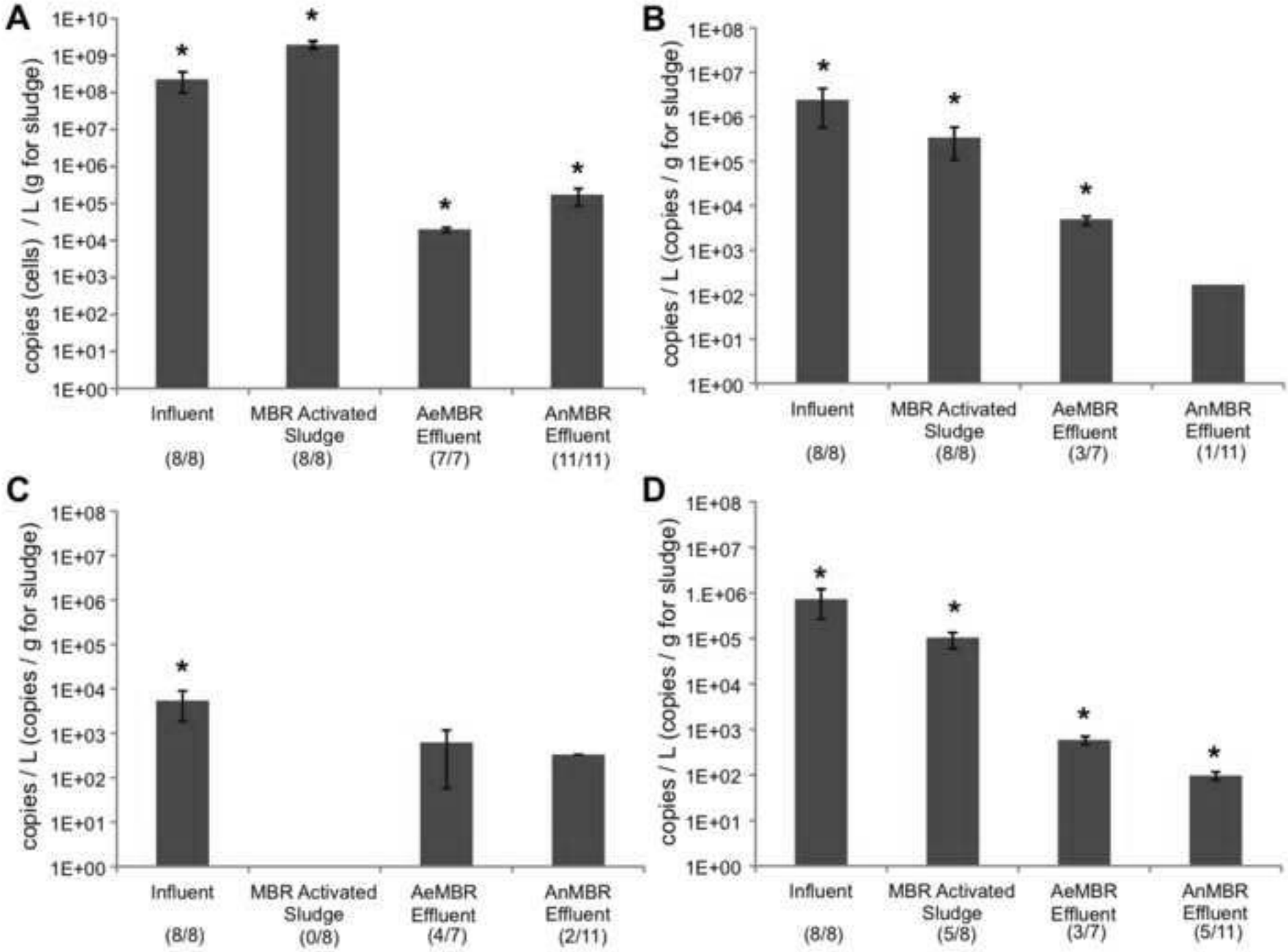

Figure 3

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