Metagenomic and Resistome Analysis of a Full-Scale Municipal Wastewater Treatment Plant in Singapore Containing Membrane Bioreactors

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Reclaimed water provides a water supply alternative to address problems of scarcity in urbanized cities with high living densities and limited natural water resources. In this study, wastewater metagenomes from 6 stages of a wastewater treatment plant (WWTP) integrating conventional and membrane bioreactor (MBR) treatment were evaluated for diversity of antibiotic resistance genes (ARGs) and bacteria, and relative abundance of class 1 integrase integrases (intI1). ARGs conferring resistance to 12 classes of antibiotics (ARG types) persisted through the treatment stages, which included genes that confer resistance to aminoglycoside [aadA, aph(6)-I, aph(3)-I, aac(6’)-I, aac(6’)-II, ant(2’’)-I], beta-lactams [class A, class C, class D beta-lactamasmes (bla\textsuperscript{KPC}, bla\textsuperscript{OXA}), chloramphenicol (acetyltransferase, exporters, floR, cmlA), fosmidomycin (rosAB), macrolide-lincosamide-streptogramin (macAB, ereA, ermFB), multidrug resistance (subunits of transporters), polymyxin (armA), quinolone (qnrS), rifamycin (arr), sulfonamide (sul1, sul2), and tetracycline (tetM, tetG, tetE, tet36, tet39, tetR, tet43, tetQ, tetX). Although the ARG subtypes in sludge and MBR effluents reduced in diversity relative to the influent, clinically relevant beta lactamases (i.e., bla\textsuperscript{KPC}, bla\textsuperscript{OXA}) were detected, casting light on other potential point sources of ARG dissemination within the wastewater treatment process. To gain a deeper insight into the types of bacteria that may survive the MBR removal process, genome bins were recovered from metagenomic data of MBR effluents. A total of 101 close to complete draft genomes were assembled and annotated to reveal a variety of bacteria bearing metal resistance genes and ARGs in the MBR effluent. Three bins in particular were affiliated to Mycobacterium smegmatis, Acinetobacter lwofii, and Flavobacterium psychrophila, and carried acquired ARGs aac(2’’)-Ib, bla\textsuperscript{OXA} -278, and tet36 respectively. In terms of indicator organisms, cumulative log removal values (LRV) of Escherichia coli, Enterococci, and P. aeruginosa...
INTRODUCTION

Wastewater treatment plants (WWTP) receive large volumes of sewage that are enriched in nutrients, chemicals, and bacteria originating from human and animal wastes (Rizzo et al., 2013; Lood et al., 2017). Prevailing resistome datasets point to WWTP as hotspots of antibiotic resistance genes (ARG) and mobile genetic elements (MGE), functioning as point sources for antimicrobial resistance dissemination due to the release of large volumes of treated effluent into the environment (Yang et al., 2014; Ju et al., 2015; Li A. D. et al., 2015; Bengtsson-Palme et al., 2016; Guo et al., 2017). A recent large scale analysis of 484 publicly available metagenomes concluded that ARG abundance has strong correlation to fecal pollution (Antti et al., 2018). Hence, it is important to determine if the treatment technology utilized in municipal WWTP is sufficient for removing indicator organisms in effluents at an efficiency that meets public health guidelines to minimize risks, and evaluate the performance of ARG removal. Some studies show that conventional urban WWTP such as suspended-growth processes (activated sludge) have low removal rates of antibiotic resistant bacteria (ARB) and ARGs (Martins da Costa et al., 2006), and in some cases have higher prevalence of antibiotic resistant pathogens in treated effluents (Ferreira da Silva et al., 2007; Luczkiewicz et al., 2010; Al-Jassim et al., 2015) compared to raw influent. This fuels questions regarding the effectiveness of conventional wastewater treatment processes to reduce these emerging contaminants in order to allay public health concerns.

Membrane filtration, although costly, is efficient at removing a high proportion of bacteria due to the association of microbial communities with solid particles in wastewater (Lood et al., 2017). Harb and Hong (2017) reported that membrane bioreactor (MBR) filtering resulted in high log reduction values which is consistent with those in another study (Le et al., 2018). WWTP trains that include a combination of suspended-growth and settling process prior to membrane bioreactor (MBR) treatment are capable of high removal efficiencies (>70%) of certain types of antibiotics (e.g., beta-lactams, glycopeptides, fluoroquinolones) with other antibiotics persisting (e.g., lincomycin, trimethoprim) past MBR treatment (Tran et al., 2016). The exposure of wastewater microbiomes to sub-inhibitory concentrations of antibiotics within different compartments of the wastewater treatment process may allow selection and mutations within the microbial community that enable them to exhibit phenotypic resistance (Chait et al., 2016; Singer et al., 2016). Studies have shown correlations between heavy metals, MGEs [e.g., class 1 integron integrase gene (intI1)] and sul genes, suggesting that the presence of heavy metals in WWTP play a role in co-selecting for ARGs (Di Cesare et al., 2016). While MBR systems are efficient at removing indicator bacteria such as Enterococci and Escherichia coli (Francy et al., 2012; Hai et al., 2014), complete removal of all bacteria is still not achieved (Jong et al., 2010; Trinh et al., 2012; van den Akker et al., 2014; Harb and Hong, 2017). It remains a challenge to assess the potential risk associated with reuse of MBR effluents due to lack of information of the types of bacteria that might slip through the treatment process. Furthermore, a recent metagenomic study of resistomes in 4 full-scale water reclamation plants in the United States showed elevated levels of certain ARGs in final effluents (Garner et al., 2018).

The purpose of this study is to examine shifts in the resistome profiles and microbial diversity in a full scale urban WWTP that employs both conventional and MBR for treatment of municipal sewage. Additionally, the removal of indicator organisms was assessed at various stages of the treatment train to determine the performance of each process, and the resultant concentrations of fecal contamination remaining in final effluents. To achieve this, wastewater samples were collected from each stage of the treatment train of a local WWTP during six time points at different intervals over a 12-month timeframe. Utilizing metagenomics sequencing and in silico analyses, resistomes and microbial composition of effluents from each sampled stage of the WWTP was profiled to evaluate the types of ARGs that remain after the treatment process.

MATERIALS AND METHODS

Sampling and Concentrations of Microbial Biomass

Raw sewage and treated wastewater samples were collected from five locations through the treatment train of a municipal WWTP in Singapore: influent (INF), primary settling tank (PST), secondary settling tank (SST), membrane bioreactor (MBR), and wet well (WW). Also, recycled activated solids (SLUDGE) was collected from a Modified Ludzack-ETtinger (MLE) bioreactor. Samples were collected at six different time points: October 2016, November 2016, January 2017, March 2017, May 2017, August 2017. The water reclamation process and sampling locations are

Abbreviations: ARB, antibiotic resistant bacteria; ARG, antibiotic resistant gene; WWTP, wastewater treatment plant; LRV, log removal value; INF, influent; PST, primary settling tank; SST, secondary settling tank; MBR, membrane bioreactor; WW, wet well; SLUDGE, recycle activated sludge; NEA, National Environment Agency; U.S. EPA, United States Environmental Protection Agency.
Measuring Concentrations of Indicator Organisms

To estimate the level of fecal contamination and determine whether wastewaters met microbiological criteria for recreational purposes (NEA Singapore, 2018), traditional bacterial indicators (E. coli, Enterococcus spp., P. aeruginosa) were assayed via Colilert, Enterolert, and Pseudalert kits (IDEXX Laboratories Inc., USA) following the manufacturer’s instructions with slight modification. Briefly, 100 mL of wastewater sample and 10-fold serially diluted samples were prepared by mixing the respective kit reagents in provided sterile bags and poured into a multi-well tray (Quanti-Tray 2000). All kits were incubated over 24 h at temperatures of 37°C for Colilert and Pseudalert samples, and 44°C for Enterolert samples. The trays were visualized under long-wave ultraviolet light (365 nm) and positive wells producing a fluorescence was counted and expressed as the most probable number (MPN) per 100 mL of water sample (MPN/100 mL).

Calculation of Log Removal Values (LRV) for Indicator Organisms

The removal efficiency of each indicator organism through the treatment process was expressed as log removal values (LRV) calculated using the following formula:

$$LRV = \log_{10}(C_{IN}) - \log_{10}(C_{OUT})$$

where $C_{IN}$ represents the concentration in the inlet wastewater source and $C_{OUT}$ represents the concentration in the effluent of the treatment stage. For effluent samples that did not contain detectable concentrations of indicators ($<1$ MPN/100 mL), a “zero” value was substituted in order to calculate LRV representative of the actual removal.

DNA Extraction

Nucleic acids were extracted from total wastewater biomass concentrated on 0.22 µm cellulose nitrate membrane filters (see section Sampling and Concentrations of Microbial Biomass) and 0.5 g (wet weight) of SLUDGE samples using the PowerSoil DNA Isolation Kit (Qiagen, Netherlands) according to the manufacturer’s recommendations. The quality and quantity of
DNA extracted were determined using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific, United States) and extracted DNA was run on a 1% agarose gel to ensure that samples were not degraded.

**Library Preparation and Sequencing**

Extracted DNA was sent to the Singapore Centre of Environmental Life Sciences and Engineering (SCELSE) where library preparation and sequencing was performed on the Illumina HiSeq2500 sequencer as stated in Ng et al. (2017). Metagenomic datasets were deposited into the NCBI short read archive (SRA) under BioProject accession PRJNA438174 (Table S1).

**Taxonomic Assignment and Identification of ARGs and Integron Genes**

All paired end raw reads were removed of sequence adaptors and low quality reads using bbmaps of the BBTools packages (https://sourceforge.net/projects/bbmap/). Taxonomic assignment of clean reads was done using Metaxa2 2.2 beta 10 (Bengtsson-Palme et al., 2015) with the default parameters, and results were analyzed using MEGAN 6 (Huson et al., 2016) for microbial diversity analysis. ARGs-OAP platform (http://smile.hku.hk/SARGs), an online analysis pipeline developed by Yang et al. (2016), was used for antibiotic resistance gene detection through interrogation against A Structured ARG reference database (SARG). SARG was constructed by extracting unique ARG sequences from the commonly used Antibiotic Resistance Database (ARDB) and the Comprehensive Antibiotic Resistance Database (CARD). ARG-like sequences were identified using the pipeline and sorted into subtypes (e.g., tetA, tetX, tetY) and types (e.g., tetracycline) with relative abundances measured by normalizing the number of assigned ARG reads to 16S rRNA genes in the metagenomic dataset for each sample. Cleaned reads were interrogated against integron-like sequences extracted from the INTEGRALL database (Moura et al., 2009), and relative abundance was expressed by normalizing the number of integron assigned reads to the number of 16S rRNA genes assigned reads for each sample. A log (x + 1) transformation was applied to integron relative abundance values and an unpaired Student's t-test was used to determine statistical differences (P < 0.05) between the different treatment steps.

**Statistical Analysis**

To construct heatplots of the relative abundance of ARG subtypes and types in each sample, data was normalized using a log (x + 1) transformation and plotted using the online web tool ClustVis (https://biit.cs.ut.ee/clustvis/). Primer v7 (Clarke and Gorley, 2015) was used to analyze clustering patterns of ARGs and microbial community structure (at the genus level). A log (x + 1) transformation was used to normalize datasets and a resemblance matrix was calculated using a Bray-Curtis analysis. Principal Coordinate Analysis (PCO) was used to illustrate clustering patterns of samples and an Analysis of Similarity (ANOSIM) procedure using 999 iterations were used to test for significance of the clustered groups. Only ARGs or microbial taxa that had a Pearson correlation of >0.8 within clustered groups were represented as vectors in PCO plots.

**Metagenomic Binning of MBR Effluent**

All clean metagenomics reads from the MBR effluent were assembled into contigs using Megahit (Li D. et al., 2015). Genomic binning was conducted using MetaBinner 2 (Kang et al., 2015) and MaxBin (Wu et al., 2014), after which genome refinement was done using MetaWRAP (Uritskiy et al., 2018). The quality of genomes recovered was measured for completeness and contamination using CheckM (Parks et al., 2014). To identify genomes containing acquired ARGs, each bin was searched against Resfinder 3.0 (https://cge.cbs.dtu.dk/services/ResFinder/) at a selected threshold of 90% and minimum query length of 60%. Gene calling and functional annotation of genome bins were performed using RAST (Rapid Annotation Using Subsystem Technology, Overbeek et al., 2014) available online (http://rast.nmpdr.org/).

**RESULTS**

**Removal Efficiency of Indicator Organisms**

Although mean concentrations for each indicator were abundant in raw sewage (E. coli $1.3 \times 10^5 \pm 8.9 \times 10^5$ MPN/100 mL, Enterococci $1.1 \times 10^6 \pm 5.6 \times 10^6$, P. aeruginosa $4.7 \times 10^6 \pm 4.8 \times 10^6$ MPN/100 mL), sedimentation during PST stages resulted in only slight reduction ($<1.00$) of E. coli ($2.3 \times 10^3 \pm 2.3 \times 10^3$ MPN/100 mL) and Enterococci ($6.8 \times 10^2 \pm 2.2 \times 10^2$ MPN/100 mL), as well as P. aeruginosa ($4.7 \times 10^6 \pm 4.8 \times 10^6$ MPN/100 mL) (Table 1). Suspended-growth AO-A bioreactors followed by SST resulted in mean LRV of 1.35 for E. coli and 1.77 for P. aeruginosa, and 1.81 for Enterococci. However, secondary effluent from this train contained $10^4$ to $10^5$ MPN/100 mL of each indicator organism. The majority of indicator removal occurred during MBR secondary treatment, as this stage resulted in mean LRV of 6.86 $\pm 0.80$ for E. coli, 5.98 $\pm 0.22$ for Enterococci and 6.03 $\pm 0.58$ for P. aeruginosa. Effluent from MBR did not contain detectable concentrations of E. coli or Enterococci ($<1$ MPN/100 mL), but contained P. aeruginosa at a mean concentration of $4.0 \pm 2.8$ MPN/100 mL.

**Occurrence of ARG Types and Subtypes in the Treatment Train**

The average diversity of ARG subtypes was higher in the INF, SST, and MBR (1.350), INF (1.106), and PST (0.857) while the WW, SST, and SLUDGE displayed 1.12, 0.69, and 0.22 for Enterococci (6.8 $\times 10^2$ MPN/100 mL), but contained P. aeruginosa at a mean concentration of $4.0 \pm 2.8$ MPN/100 mL.
resistance to beta-lactam antibiotics. The 3 most abundant ARG subtypes belonged to the ARG type beta-lactam (class A, class C, class D beta-lactamases), multidrug resistance (subunits of transporters), polymyxin (arnA), quinolone (qnrS), rifamycin (arr), sulfonamide (sul1, sul2), and tetracycline (tetM, tetG, tetE, tet36, tet39, tetR, tet43, tetQ, tetX) (Figure 3). A Principal Coordinate Analysis (PCO) indicated that at the ARG subtype level, resistome profiles of INF, SST and WW clustered and were different from that of SLUDGE and MBR (ANOSIM test \( R = 0.849, P = 0.001, \) Figure 3). A Pearson correlation (\( R > 0.85, \) \( P = 0.001 \)) showed that ARG subtypes, class A beta-lactamase, cAMP regulatory proteins, and kasugamycin resistance protein (ksgA) correlated with the INF, SST, and WW cluster, while multidrug efflux pumps (oprN, mexEF, mdtB) correlated with the MBR samples.

### The Shared Resistome

Wastewaters from all six locations along the treatment train shared a core resistome of 100 ARG subtypes belonging to 12 ARG types (Figure 3). There were 12 main ARG types that persisted through the treatment stages, which included genes that confer resistance to aminoglycoside (aadA, aph(6)-I, aph(3’)-I, aac(6’)-I, aac(6’)-II, ant(2’)-I), beta-lactams [class A, class C, class D beta-lactamases (blaOXA)], chloramphenicol (acetyltransferase, exporters, floR, cmIA), fosfomycin (rosAB), MLS (macAB, ereA, ermFB), multidrug resistance (subunits of transporters), polymyxin (arnA), quinolone (qnrS), rifamycin (arr), sulfonamide (sul1, sul2), and tetracycline (tetM, tetG, tetE, tet36, tet39, tetR, tet43, tetQ, tetX) (Figure S1). A Heatmap of the ARG types across all samples is represented in Figure 2. ARG subtypes associated with multidrug resistance and resistance to aminoglycoside (aac, aad, ant, aph), bacitracin (bacA), beta-lactams (blaCR, blaER, blaOXA, blaIMP, blaKPC, blaAEC, blaPEC), chloramphenicol (chloramphenicol exporters, cmIA, floR), fosfomycin (rosAB), MLS (ere, erm, macAB, mssA), polymyxin (arnA), quinolone (qnrS), rifamycin (arr), sulfonamide (sul1, sul2), tetracycline (tet36, tet39, tet41, tet43, tetA, tetC, tetE, tetG, tetL, tetQ, tetR, tetV, tetX), trimethoprim (dfrAB) and vancomycin (vanRSX) were detected in the MBR effluent.

### Relative Abundance of Class 1 Integrin-Integrate Genes (intI1)

The relative abundance of intI1 genes was highest in INF (33 ± 9) and PST (34 ± 8), compared to the other points further down the treatment train (WW 28 ± 5, SST 24 ± 5, MBR 16 ± 13, SLUDGE 14 ± 7) (Figure 4). There were no significant differences from the INF to PST, however there was a significant (\( P < 0.05 \)) reduction in relative abundance values from PST to SST, and SST to SLUDGE (Figure 4). There were no significant reductions in the relative abundance of intI1 genes from SST to MBR.

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TABLE 1 | Log removal values (LRV) of indicator organisms along the wastewater treatment train.

|          | MPN/100 mL | Log value (log10) | LRV |
|----------|------------|------------------|-----|
|          | INF PST SST MBR | INF PST SST MBR | INF→PST INF→SST INF→MBR |
| Entero| Oct 2016 | 1.41 x 10^7 | 5.92 x 10^5 | 4.98 | 3.12 | 2.90 | 1.75 | 0.10 | 1.43 | 7.15 |
| Enterococci | Nov 2016 | 1.33 x 10^7 | 3.87 x 10^5 | 3.87 | 3.75 | 3.50 | 2.10 | 0.10 | 1.63 | 7.22 |
| E. coli | Jan 2017 | 1.45 x 10^7 | 3.13 x 10^5 | 3.13 | 3.00 | 2.75 | 1.70 | 0.11 | 1.66 | 7.16 |
| Enterococci | Mar 2017 | 8.84 x 10^6 | 2.70 x 10^5 | 2.70 | 2.50 | 2.25 | 1.80 | 0.17 | 1.52 | 6.96 |
| Enterococci | May 2017 | 2.70 x 10^7 | 1.90 x 10^5 | 1.90 | 1.75 | 1.65 | 1.00 | -0.13 | -0.10 | 6.10 |
| Enterococci | Aug 2017 | 1.87 x 10^7 | 3.87 x 10^5 | 3.87 | 3.75 | 3.50 | 2.10 | 0.10 | 1.43 | 7.15 |

INF: influent; PST, primary settling tank effluent; SST, secondary settling tank effluent; MBR, membrane bioreactor effluent.
Taxonomic Composition

The Shannon-Weaver and Simpson reciprocal indices were used to assess the microbial diversity for each sample. The average values showed SLUDGE had the greatest bacterial diversity followed by SST, WW, INF, PST, and MBR (Table S3). A Principal Coordinate Analysis showed that bacterial community structure was divided into two main clusters, one consisting of INF, PST, SST, MBR and WW and another represented by SLUDGE samples (ANOSIM test \( R = 0.357, P = 0.01 \), Figure 5). *Pseudomonas, Bacteroides, Aeromonas, Prevotella,* and *Cloacibacterium* dominated the first cluster in wastewater samples. However, a higher prevalence of *Alteromonadaceae, Pedobacter, Gemmatimonas,* and *Flexibacter* differentiated SLUDGE from the rest of the samples (Pearson correlation \( R > 0.8 \), Figure 5B). A core microbiome present across all samples was composed of *Pseudomonas, Acinetobacter, Cloacibacterium, Acidovorax, Comamonas, Flavobacterium,* and *Azospira. Pseudomonas* (33%), *Acinetobacter* (25%), *Varivorax* (12%), *Comamonas* (5%), *Thermomonas* (4%), *Acidovorax* (3%), and *Delftia* (3%) persisted in post-MBR treated effluent, while *Cloacibacterium* (24%), *Pseudomonas* (11%), *Aeromonas* (8%), *Arcobacter* (6%), *Flavobacterium* (5%), *Bacteroides* (5%), *Acinetobacter* (5%) persisted in SST treated effluent (Figure 5A, Table S4). The WW bacterial community comprised of taxa that were present in SST and MBR effluent with a dominance of *Cloacibacterium* (26%), *Pseudomonas* (8%), *Flavobacterium* (7%), *Acinetobacter* (6%), and *Acidovorax* (2%).

Binned Bacterial Genomes Recovered From MBR Effluent

A total of 101 microbial genomes were recovered and assembled from the MBR treated effluent, and a criteria of >70% completeness and a contamination of <9.6% was selected. The binned genomes were assigned to 59 unique bacterial species based on taxonomic assignments provided by RAST (Table S5). The dominant genera representing at least 3% of the total number of bins included *Magnetospirillum, Acidovorax, Nitrospira, Rhodospirillum, Sphingopyxis, Flavobacterium, Acinetobacteria, Chitinophagaceae, Dechloromonas,* *Janthinobacterium, Mycobacterium,* and *Novosphingobium* (Table S5). Other potential fish pathogens such as *Flavobacterium* spp., *Chryseobacterium* spp., and a chlamydial bacterial endosymbiont in *Acanthamoebae, Parachlamydiae* spp., and the opportunistic human pathogen *Comomonas* spp. represented at least one genome in the total number of bins identified. Annotation of binned genomes indicated that functional genes related to antibiotic resistance (fluoroquinolone resistance, aminoglycoside adenylyltransferase, tetracycline resistance and ribosomal protection type, beta-lactamase, fosfomycin resistance, streptothricin resistance, multidrug resistance efflux pumps, MexC-MexD-OprJ multidrug efflux systems) and metal resistance (copper homeostasis, cobalt-zinc-cadmium resistance, zinc resistance, mercuric reductase/resistance operon, arsenic resistance, chromium compound resistance) were present in the genomes of bacteria in the MBR effluent.
FIGURE 3 | Shared resistomes along the wastewater treatment train. Overlapping ARGs detected at (A) all six-, (B) five-, (C) four-, (D) three-, and (E) two- treatment stages. The ARG was considered detected as long as it was present in at least one collected time point within the treatment stage. (F) A PCO plot of the ARG subtypes. Vectors represent ARGs that had Pearson correlation of >0.8.
A query against the Resfinder database identified 3 bins (bins 19, 23, 76) harboring acquired ARGs which showed high homology to \textit{aac(2')-Ib, bla\textsubscript{OXA-278}, tet36}, respectively (Figure 6). These three bins were affiliated to \textit{Mycobacterium smegmatis, Acinetobacter Iwoffii, Flavobacterium psychrophila} that carried other genes associated with metal and antibiotic resistance (Table S6).

**DISCUSSION**

Water management is one of the cornerstones of sustainable urban development; hence, knowing the efficiency of wastewater treatment technology employed in WWTPs is essential to understand how the effluents may affect downstream processes and the urban water cycle. According to European legislation, traditional parameters such as total suspended solids, chemical oxygen demand, biochemical oxygen demand, ammonia, nitrate, total phosphorus, and fecal coliforms are used to assess the quality of treated wastewaters (EEC, 1998). The indiscriminant usage of antibiotics in human and animals has perpetuated the antimicrobial resistance problem globally (WHO, 2014; Ventola, 2015). Wastewater discharges originating from hospitals and domestic sources carry fecal pathogens, as well as ARB and ARGs which are emerging environmental contaminants (Rizzo et al., 2013; Le et al., 2016; Ng et al., 2017; An et al., 2018; Gupta et al., 2018; Haller et al., 2018; Lorenzo et al., 2018; Manaia et al., 2018). If wastewaters are not treated using appropriate technology, environments receiving wastewater discharge may be impacted (Rodriguez-Mozaz et al., 2015; Proia et al., 2018; Sabri et al., 2018), particularly where fecal indicator concentrations in effluents are high (Antti et al., 2018).

In this full-scale study of a municipal WWTP, cumulative LRV of indicator organisms (\textit{E. coli, Enterococci, P. aeruginosa}) from INF to post-SST was 0–2.4, and 5.3–7.4 post-MBR, which validates a greater removal capacity via MBR as evident in other studies (Harb and Hong, 2017; Zhu et al., 2018). The fecal coliform values were comparable to wastewater treatment plants in Sweden and Italy that apply conventional and MBR treatments (Ottoson et al., 2006). The LRV of \textit{E. coli} reported in our study was slightly higher than that of the Swedish study, as well as LRV median values (5.9) of nine different wastewater MBR systems in California (Hirani et al., 2012). Still, LRV of \textit{E. coli} (7) and \textit{Enterococci} (6) were comparable to another study with a full scale MBR system in a municipal WWTP in Italy (Zanetti et al., 2010). \textit{E. coli} and \textit{Enterococci} in the MBR effluent were below the detection limit (<1MPN/100 mL), which meets the U.S. EPA guidelines for recreational purposes, and within reported limits used for agriculture reuse for surface or spray irrigation of food crop intended for human consumption (U. S. EPA, 1986, 2012).

Despite the absence of \textit{E. coli} and \textit{Enterococci} in MBR effluents, low abundance of viable \textit{P. aeruginosa} were present. The microbial community profile from INF, PST, to SST appear fairly stable with a slight shift observed after MBR treatment, where certain taxa continued to predominated and others waned. The MBR effluent had a microbial community profile dominated by species of \textit{Pseudomonas, Acinetobacter, Varivorax, Comamonas, Thermomonos, Acidovorax, Delftia, Sphingomonas, Chryseobacterium, Azospira, and Flavobacteria}. The same genera of bacteria have been described post MBR treated effluent in several other studies (Grijalbo et al., 2015; Harb and Hong, 2017; Liu et al., 2017). Of these taxa, \textit{Pseudomonas} and \textit{Acinetobacter} are pathogen-associated genera and their detectable levels in MBR treated effluents may pose problems in receiving waters or enable regrowth in storage tanks. Furthermore, the ARG profile of MBR effluents show high relative abundance of fosmidomycin, beta lactam and puromycin ARG types. Also, MBR profiles indicate the persistence of a diversity of 100 ARG subtypes detected throughout the treatment process with resistance to aminoglycoside, beta-lactams (class A, class C, class D beta-lactamases) chloramphenicol, fosfomycin, MLS, polymyxin, quinolone, rifamycin (\textit{arr}), sulfonamide, tetracycline, and \textit{intI1} genes (associated with horizontal gene transfer). Detection of sulfonamide \textit{((sul1, sul2)}, tetracycline \textit{(tetC, tetX, tetG)}, MLS \textit{(ereA)} resistant genes and \textit{intI1} in our study has also been observed in MBR effluents of municipal wastewater treatment plants in China (Du et al., 2015; Zhu et al., 2018). Among the beta lactamases, variants of the \textit{bla\textsubscript{OXA}} genotypes (class D beta lactamase) were detected across the treatment process, including in the sludge. A variety ARG subtypes belonging to 14 different ARG types (multidrug resistance, polymyxin, quinolone, rifamycin, fosfomycin, chloramphenicol, beta lactam, bacitracin aminoglycoside, sulfonamide, tetracycline, MLS, vancomycin, trimethoprim) were detected in sludge samples which underscores problems of the application of sludge for organic farming purposes. Another study by Calero-Cáceres et al. (2014) provided evidence of sludge being a potentially important source of ARGs.

The ubiquity of different types of \textit{bla\textsubscript{OXA}} is consistent with previous findings in hospital wastewater effluents and municipal wastewater samples in Singapore (Ng et al., 2017). Variants of class A (\textit{bla\textsubscript{KPC}, bla\textsubscript{PER}}), class B (\textit{bla\textsubscript{GOB, bla\textsubscript{LRA}}}), and class C (\textit{bla\textsubscript{PPC}}) beta lactamases were detected in INF and MBR effluents, which confer resistance to beta lactam antibiotics such as cephalosporin and carbapenems. These antibiotics are of clinical importance and considered as last resort antibiotics in the
treatment of Gram-negative infections (Nordmann et al., 2011; WHO, 2016). This result suggests that even with advanced MBR treatment, these ARG subtypes persist and further treatment and monitoring would be necessary to prevent dissemination. Detection of \textit{blaPDC}, a chromosomally inducible \textit{Pseudomonas}-derived cephalosporinase (PDC), and the presence of viable \textit{P. aeruginosa} cells in MBR effluent is a basis for \textit{P. aeruginosa} playing an important role in the carriage and dissemination of antimicrobial resistance in post MBR treated effluent. Although low concentrations of viable \textit{P. aeruginosa} (<8 MPN/100 mL) were measured in the MBR effluent, their biofilm forming ability and antibiotic tolerant properties within a biofilm community (Spoering and Lewis, 2001; Harrison et al., 2005) could allow them to colonize surfaces in the post MBR wastewater treatment.
train providing a protective environment for other ARB to thrive. Moreover, plasmid bearing blaKPC in P. aeruginosa strains have been described in local hospital wastewaters (Ng et al., 2017; Haller et al., 2018); providing a basis for prioritizing the monitoring of this ARB in wastewater effluents.

To infer the implications of the detectable array of ARGs in the MBR effluent, metagenomic binning was used to assign ARGs to specific taxa. Only a few binned genomes from the MBR effluent belonged to genera associated with opportunistic pathogens, consistent with taxa (Acinetobacter, Mycobacterium, Stenotrophomonas, Pseudomonas) also observed in a 16S rRNA-based microbial classification study of municipal water effluent by Harb and Hong (2017). Two other genome bins affiliated to Flavobacterium (Flavobacterium johnsoniae, Flavobacterium psychrophilum) and Chryseobacterium (Chryseobacterium gleum), known to cause infections in fish and occasionally humans (Loch and Faisal, 2015) were recovered from the MBR effluent metagenomes. Metal resistance genes and intrinsic ARGs were annotated in binned bacterial genomes of the MBR effluents. This provides new insights and a direct genetic link of members within the microbial consortia with resistance mechanisms which allow them to protect themselves and survive in the presence of antimicrobials or heavy metals. Only three acquired ARGs were detected in the genome bins of M. smegmatis, A. Iwoffii, F. psychrophila and the lack of the identification of more acquired ARGs could be attributed to the difficulties in assigning plasmid sequences to genomic bins. These bacteria are not considered priority pathogens on WHO’s published list of antibiotic-resistant “priority pathogens” (Lawe-Davis and Bennett, 2017). Despite this, it should be emphasized that A. Iwoffii, which is ubiquitous in the environment is an emerging multidrug resistant pathogen in neonatal sepsis (Mittal et al., 2015). Furthermore, ARGs encoding for resistance against cephalosporins, macrolides, polymyxins, quinolones, and aminoglycosides, that are categorized as critically important antimicrobials for human medicine by WHO (2016), persisted throughout the wastewater treatment process. Monitoring the fate of these genes, beyond the WWTP and into receiving waters, such as the blaOXA genes that confer resistance to cephalosporins, could be used to track the extent of dissemination and the evaluate potential risks.

Shifts in community composition were observed at each treatment stage, with the SLUDGE samples having the highest diversity enriched in bacteria involved in the removal of nitrogen (Nitrosira, Nitromonas) and phosphorus (Gemmatimonas), formation of bioflocs (Chloroflexaceae, Caldilinea), and other taxa involved in degradation pathways (Porphyromonadaceae, Haliangium) which are typically present in activated sludge (Ferrera and Sanchez, 2016; Shchegolkova et al., 2016) and a good indication of adequate wastewater treatment. Unlike the other samples where beta lactams were the dominant ARG type, the SLUDGE displayed a different profile of tetracycline (tetX), bacitracin (bacA), and multidrug resistance (ompR, acrB) genes.

The reduction in the diversity of ARG subtypes and concentrations of fecal indicators along the treatment train of a full-scale municipal wastewater system is a clear indication that MBR as a treatment strategy is effective at removing raw-sewage associated bacteria and most ARGs in the influent, although removal of P. aeruginosa was not as effective. In another study, Zhu et al. (2018) reported that the development of dense membrane fouling layers reinforces the membrane itself creating a dual barrier that prevents leakage of ARGs from the membrane module. Metagenomic binning results indicate that remaining ARGs in the MBR effluent (that were not assigned to any bin) could possibly be extracellular fragmented DNA, plasmid- or phage-associated. The relevance

**FIGURE 6** A comparison of binned genomic regions in MBR effluents harboring acquired ARGs. Genomic bin numbers are seen in parenthesis. Sets of genes with similar sequences are grouped by color with those in red representing acquired ARGs identified by Resfinder. Corresponding ARG homologs present in similar organisms were compared against the binned genomic regions.
of exogenous ARGs is unknown and it remains a challenge to understand the fate, transport, and health impact that ARGs have on reclaimed water (Hong et al., 2018). Apart from ARG removal, MBR systems have demonstrated higher removal rates of antibiotics and antimicrobials from wastewater influents in contrast to conventional treatment options (Wang et al., 2015; Tran et al., 2016). Although there are currently no benchmarks for maximum admissible values of ARB and ARGs in treated wastewater, our results support the view that MBR is a superior option of treatment for water reuse compared to relying solely on conventional treatment processes that incorporate activated mixed liquors, where effluents still contain relatively high levels of ARGs and indicator organisms. However it should be noted that the MGE, int11, did not show significant rates of removal with MBR treatment.

**AUTHOR CONTRIBUTIONS**

CN, HC, BS, LH, and FC conceptualized and designed the study. CN, HC, BS, LH, and FC processed samples and assisted with data collection. CN, BT, X-TJ, XG, and TZ provided technical expertise in sequence data analysis, assembly, and interpretation. CN and KG wrote the manuscript. KG and BT helped obtain funding support for the work.

**FUNDING**

This research was funded by the Ministry of Education (MOE), Academic Research Fund (AcRF) Tier 2 grant (reference: MOE2015-T2-2-130), and The Public Utilities Board ( PUB) Research & Development Project (reference: RND-WAQU-1510-0013). We thank the National University of Singapore (NUS) for supporting this research.

**ACKNOWLEDGMENTS**

We would like to thank Wu Zhixin for helping with the field work, and Chang Siaoyun for coordinating the sampling schedule at the water reclamation plant.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00172/full#supplementary-material

**REFERENCES**

Al-Jassim, N., Ansari, M. I., Harb, M., and Hong, P. Y. (2015). Removal of bacterial contaminants and antibiotic resistance genes by conventional wastewater treatment processes in Saudi Arabia: is the treated wastewater safe to reuse for agricultural irrigation? Water Res. 73, 277–290. doi: 10.1016/j.watres.2015.01.032

An, X. L., Su, J. Q., Li, B., Ouyang, W. Y., Zhao, Y., Chen, Q. L., et al. (2018). Tracking antibiotic resistome during wastewater treatment using high throughput quantitative PCR. Environ. Int. 117, 146–153. doi: 10.1016/j.envint.2018.05.011

Antti, K., Katarina, P., and Joakim, L. D. G. (2018). Fecal pollution explains antibiotic resistance gene abundances in anthropogenically impacted environments. *bioRxiv*. doi: 10.1101/341487

Bengtsson-Palme, J., Hammaren, R., Pal, C., Ostman, M., Bjorlenius, B., Flach, C. F., et al. (2016). Elucidating selection processes for antibiotic resistance in sewage treatment plants using metagenomics. *Sci. Tot. Environ.* 572, 697–712. doi: 10.1016/j.scitotenv.2016.06.228

Bengtsson-Palme, J., Hartmann, M., Eriksson, K. M., Pal, C., Thorell, K., Larsson, D. G. J., et al. (2015). Metaxa2: improved identification and taxonomic classification of small and large subunit rRNA in metagenomic data. *Mol. Ecol. Resour.* 15, 1403–1414. doi: 10.1111/1755-0998.12399

Calero-Cáceres, W., Melgarejo, A., Colomer-Lluch, M., Stoll, C., Lucena, F., Jofre, J., et al. (2014). Sludge as a potential important source of antibiotic resistance genes in both the bacterial and bacteriophage fractions. *Environ. Sci. Technol.* 48, 77662–77671. doi: 10.1021/es503851s

Chait, R., Palmer, A. C., Yelin, I., and Kishony, R. (2016). Pervasive selection for and against antibiotic resistance inhomogeneous multistress environments. *Nat. Commun.* 7:10333. doi: 10.1038/ncomms10333

Clarke, K. R., and Gorley, R. N. (2015). PRIMER v7: User Manual/Tutorial. Plymouth: PRIMER-E.

Di Cesare, A., Eckert, E. M., D’Urso, S., Bertoni, R., Gillan, D. C., Wattiez, R., et al. (2016). Co-occurrence of integrase 1, antibiotic and heavy metal resistance genes in municipal wastewater treatment plants. *Water Res.* 94, 208–214. doi: 10.1016/j.watres.2016.02.049

Du, J., Geng, J., Ren, H., Ding, L., Xu, K., and Zhang, Y. (2015). Variation of antibiotic resistance genes in municipal wastewater treatment plant with A2-O-MBR system. *Environ. Sci. Pollut. Res.* 22, 3715–3726. doi: 10.1007/s11356-014-3552-x

EEC (1998). *Amending Council Directive 91/271/EEC. Commission directive 98/15/EC.*

Ferreira da Silva, M., Vaz-Moreira, I., Gonzalez-Pajuelo, M., Nunes, O. C., and Manaia, C. M. (2007). Antimicrobial resistance patterns in Enterobacteriaceae isolated from an urban waste water treatment plant. *FEMS Microb. Ecol.* 60, 166–176. doi: 10.1111/j.1574-6941.2006.00268.x

Ferrera, I., and Sanchez, O. (2016). Insights into microbial diversity in wastewater treatment systems: how far have we come? *Biotechnol. Adv.* 34, 790–802. doi: 10.1016/j.biotechadv.2016.04.003

Francy, D. S., Stelzer, E. A., Bushon, R. N., Brady, A. M. G., Williston, A. G., Riddell, K. R., et al. (2012). Comparative effectiveness of membrane bioreactors, conventional secondary treatment, and chlorine and UV disinfection to remove microorganisms from municipal wastewaters. *Water Res.* 46, 4164–4178. doi: 10.1016/j.watres.2012.04.044

Garner, E., Chen, C., Bowers, J., Englerthal, D. M., McLain, J., Edwards, M. A., et al. (2018). Metagenomic characterization of antibiotic resistance genes in full-scale reclaimed water distribution system and corresponding potable systems. *Environ. Sci. Technol.* 52, 6113–6125. doi: 10.1021/acs.est.7b05419

Grijalbo, L., Garbisu, C., Martin, I., Etxebarria, J., Gutierrez-Mañero, F. J., and Lucas Gracia, J. A. (2015). Functional diversity and dynamics of bacterial communities in a membrane bioreactor for the treatment of meta-working fluid wastewater. *J. Water Health* 13, 1006–1019. doi: 10.2166/wh.2015.079

Guo, J., Li, J., Chen, H., Bond, P. L., and Yuan, Z. (2017). Functional diversity and dynamics of bacterial communities in a membrane bioreactor for the treatment of meta-working fluid wastewater. *J. Water Health* 13, 1006–1019. doi: 10.2166/wh.2015.079

Guo, J., Li, J., Chen, H., Bond, P. L., and Yuan, Z. (2017). Metagenomic analysis reveals wastewater treatment plants as hotspots of antibiotic resistance genes and mobile genetic elements. *Water Res.* 123, 468–478. doi: 10.1016/j.watres.2017.07.002

Gupta, S. K., Shin, H., Han, D., Hur, H. G., and Unno, T. (2018). Metagenomic analysis reveals the prevalence and persistence of antibiotic- and heavy metal-resistance genes in wastewater treatment plant. *J. Microbiol.* 56, 408–415. doi: 10.1007/s12275-018-8195-z

Hai, F. I., Riley, T., Shawkat, S., Magram, S. F., and Yamamoto, K. (2014). Removal of pathogens by membrane bioreactors: a review of the mechanisms.
influencing factors and reduction in chemical disinfectant dosage. Water 6, 3603–3630. doi: 10.3390/w6123603
Hallier, L., Chen, H., Ng, C., Le, T. H., Barkham, T., Sobsey, M., et al. (2018). Occurrence and characteristics of extended-spectrum β-lactamase- and carbapenemase-producing bacteria from hospital effluents in Singapore. Sci. Total. Environ. 615, 1119–1125. doi: 10.1016/j.scitotenv.2017.09.217
Harb, M., and Hong, PY (2017). Molecular-based detection of potentially pathogenic bacteria in membrane bioreactor (MBR) systems treating municipal wastewater: a case study. Environ. Sci. Pollut. Res. Int. 24, 5370–5380. doi: 10.1007/s11356-016-8211-y
Harrison, J. J., Turner, R. J., and Ceri, H (2005). Persistier cells, the biofilm matrix and tolerance to metal cations in biofilm and planktonic Pseudomonas aeruginosa. Environ. Microbiol. 7, 981-994. doi: 10.1111/j.1462-2920.2005.00777.x
Hirani, Z. M., DeCarolis, J. F., Lehman, G., Adham, S. S., and Jangcione, J. G. (2012). Occurrence and removal of microbial indicators from municipal wastewaters by nine different MBR systems. Water Sci. Technol. 66, 865–871. doi: 10.2166/wst.2012.261
Hong, P. Y., Julian, T. R., Pype, M. L., Jiang, S. C., Graham, D., Pruden, A., et al. (2018). Reusing treated wastewater: consideration of the safety aspects associated with antibiotic-resistant bacteria and antibiotic resistance genes. Water 10:244. doi: 10.3390/w10030244
Huson, D. H., Beier, S., Flade, I., Gorska, A., El-Hadidi, M., Mitra, S., et al. (2016). MEGAN community edition–interactive exploration and analysis of large-scale microbe genome sequencing data. PLoS Comput. Biol. 12:e1004957. doi: 10.1371/journal.pcbi.1004957
Jong, J., Lee, J., Kim, J., Hyun, K., Hwang, T., Park, J., et al. (2010). The study of pathogenic microbial communities in graywater using membrane bioreactor. Desalination 250, 568–572. doi: 10.1016/j.desal.2009.09.025
Ju, F., Li, B., Ma, L., Wang, Y., Huang, D., and Zhang, T (2015). Antibiotic resistance genes and human bacterial pathogens: occurrence, removal, and enrichment in multiple sewage digesters. Water Res. 91, 1–10. doi: 10.1016/j.watres.2015.11.071
Kang, D. D., Froula, J., Egan, R., and Wang, Z. (2015). MetaBAT, an efficient tool for accurately reconstructing single genomes from complex microbial communities. PeerJ 3:e1165. doi: 10.7717/peerj.1165
Lawe-Davis, O., and Bennett, S. (2017). WHO-List of Bacteria for Which New Antibiotics are Urgently needed. WHO (2016). Removal of viruses, parasitic protozoa and microbial indicators in a conventional and membrane processes in a wastewater pilot plant. Water Res. 40, 1449–1457. doi: 10.1016/j.watres.2006.01.039
Overbeek, R., Olsen, R., Pusch, G. D., Olsen, G. J., Davis, J. T., Dis, Z. T., et al. (2014). The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). Nucleic Acids Res. 42, D206–D214. doi: 10.1093/nar/gkt1226
Park, D. H., Imelfort, M., Skennerton, C. T., Hugenholtz, P., and Tyson, G. W. (2014). Assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. Genome Res. 25, 1042–1055. doi: 10.1101/gr.163104v3
Proia, L., Anzil, A., Subirats, H., Borrego, C., Farré, M., Llorca, M., et al. (2018). Antibiotic resistance along an urban river impacted by treated wastewaters. Sci. Total Environ. 628–629, 453–466. doi: 10.1016/j.scitotenv.2018.02.083
Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., and Ploy, M. C. (2013). Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: a review. Sci. Tot. Environ. 447, 345–360. doi: 10.1016/j.scitotenv.2013.01.032
Rodriguez-Mozaz, S., Chamorro, S., Marti, E., Huerta, B., Gros, M., Sanchez-Melsis, A., et al. (2015). Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on receiving river. Water Res. 69, 234–242. doi: 10.1016/j.watres.2014.11.021
Sahri, N. A., Schmitt, H., Van der Zaan, B., Gerritsen, H. W., Zuidema, T., Rijnaarts, H. H. M., et al. (2018). Prevalence of antibiotics and antibiotic resistance genes in wastewater effluent-receiving river in the Netherlands. J. Environ. Chem. Eng. doi: 10.1016/j.jece.2018.03.004
Shegelkova, N. M., Kravsov, G. S., Belova, A. A., Dmitriev, A. A., Kharitonov, S. L., Klimina, K. M., et al. (2016). Microbial community structure of activated sludge in treatment plants with different wastewater compositions. Front. Microbiol. 7:970. doi: 10.3389/fmicb.2016.00900
Singer, A. C., Shaw, H., Rhodes, V., and Hart, A (2016). Review of antimicrobial resistance in the environment and its relevance to environmental regulators. Front. Microbiol. 7:1728. doi: 10.3389/fmicb.2016.01728
Spoering, A. L., and Lewis, K (2001). Biofilms and planktonic cells of Pseudomonas aeruginosa have similar resistance to killing by antimicrobials. J. Bacterial. 183, 6746–6751. doi: 10.1128/JB.183.23.6746-6751.2001
Tran, N. H., Chen, H., Reinhard, M., Mao, F., and Gin, K. Y. H. (2016). Occurrence and removal of multiple classes of antibiotics and antimicrobial agents in biological wastewater treatment processes. Water Res. 104, 461–472. doi: 10.1016/j.watres.2016.08.040

Ng et al. Resistomes in Singapore’s Wastewater Treatment
Trinh, T., van den Akker, B., Coleman, H., Stuetz, R., Le-Clech, P., and Khan, S. (2012). Removal of endocrine disrupting chemicals and microbial indicators by a decentralized membrane bioreactor for water reuse. J. Water Reuse Desalination 2, 67–73. doi: 10.2166/wrd.2012.010

Uritskiy, G. V., DiRuggiero, J., and Taylor, J. (2018). MetaWRAP—A Flexible Pipeline for Genome-Resolved Metagenomic Data Analysis. bioRxiv. doi: 10.1186/s40168-018-0541-1

U. S. EPA (1986). Bacteriological Ambient Water Quality Criteria for Marine and Fresh Recreational Waters. Available online at https://www.waterboards.ca.gov/water_issues/programs/tmdl/records/region_5/1986/ref2435.pdf

U. S. EPA (2012). Guidelines for Water Reuse. Washington, DC: U. S. Environmental Protection Agency.

van den Akker, B., Trinh, T., Coleman, H. M., Stuetz, R. M., Le-Clech., and Khan, S. J. (2014). Validation of a full-scale membrane bioreactor and the impact of membrane cleaning on the removal of microbial indicators. Bioresource Technol. 155, 432–437. doi: 10.1016/j.biortech.2013.12.123

Ventola, C. L. (2015). The antibiotic resistance crisis: part I: causes and threats. Pharmacy Therapeut. 40, 277–283.

Wang, J., Li, K., Wei, Y., Cheng, Y., Wei, D., and Li, M (2015). Performance and fate of organics in a pilot MBR-NF for treating antibiotic production wastewater with recycling NF concentrate. Chemosphere 121, 92–100. doi: 10.1016/j.chemosphere.2014.11.034

WHO (2014). Antimicrobial Resistance: Global Report on Surveillance. Available online at http://www.who.int/drugresistance/documents/surveillancereport/en/

WHO (2016). Critically Important Antimicrobials for Human Medicine. Available online at http://apps.who.int/iris/bitstream/handle/10665/255027/9789241512220-eng.pdf?sequence=1

Wu, Y. W., Tang, Y. H., Tringe, S. G., Simmons, B. A., and Singer, S. W. (2014). MaxBin: an automated binning method to recover individual genomes from metagenomes using an expectation-maximization algorithm. Microbiome 2:26. doi: 10.1186/2049-2618-2-26

Yang, Y., Jiang, X., Chai, B., Ma, L., Li, B., Zhang, A., et al. (2016). ARGs-OAP: online analysis pipeline for antibiotic resistance genes detection from metagenomic data using an integrated structured ARG-database. Bioinformatics 31, 2346–2351. doi: 10.1093/bioinformatics/btw136

Yang, Y., Li, B., Zou, S., Fang, H. H. P., and Zhang, T (2014). Fate of antibiotic resistance genes in sewage treatment revealed by metagenomic approach. Water Res. 62, 97–106. doi: 10.1016/j.watres.2014.05.019

Zanetti, F., De Luca, G., and Sacchetti, R. (2010). Performance of a full-scale membrane bioreactor system in treating municipal wastewater for reuse purposes. Bioresour. Technol. 101, 3768–3771. doi: 10.1016/j.biortech.2009.12.091

Zhu, Y., Wang, Y., Zhou, S., Jiang, X., Ma, X., and Liu, C (2018). Robust performance of a membrane bioreactor for removing antibiotic resistance genes exposed to antibiotics: role of membrane foulants. Water Res. 130, 139–150. doi: 10.1016/j.watres.2017.11.067

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.