Lebanese American University

Assessing intracellular and extracellular antibiotic resistance gene proliferation during the treatment of municipal wastewater by an anaerobic membrane bioreactor

By

Charbel Elkhoury

A Thesis Submitted in partial fulfillment of the requirements for the degree of Master of Science in Engineering

School of Engineering

July 2022

© 2022

Charbel Elkhoury

All Rights Reserved

LAU
لبنانية الأمريكية
Lebanese American University

THESIS APPROVAL FORM

Student Name: Charbel Elkhoury I.D. #: 201602786

Thesis Title: Assessing intracellular and extracellular antibiotic resistance gene proliferation during the treatment of municipal wastewater by an anaerobic membrane bioreactor

Program: Civil and Environmental Engineering

Department: Civil Engineering

School: Engineering

The undersigned certify that they have examined the final electronic copy of this thesis and approved it in Partial Fulfillment of the requirements for the degree of:

Master of Science in the major of Civil and Environmental Engineering

Thesis Advisor's Name Moustapha Harb	Signature [redacted]	DATE: 25 / 07 / 2022 (Day / Month / Year)
Committee Member's Name Mahmoud Wazne	Signature [redacted]	DATE: 25 / 07 / 2022 (Day / Month / Year)
Committee Member's Name Sima Tokajian	Signature [redacted]	DATE: 25 / 07 / 2022 (Day / Month / Year)

LAU
الجامعة اللبنانية الأميركية
Lebanese American University

THESIS COPYRIGHT RELEASE FORM

LEBANESE AMERICAN UNIVERSITY NON-EXCLUSIVE DISTRIBUTION LICENSE

By signing and submitting this license, you (the author(s) or copyright owner) grants the Lebanese American University (LAU) the non-exclusive right to reproduce, translate (as defined below), and/or distribute your submission (including the abstract) worldwide in print and electronic formats and in any medium, including but not limited to audio or video. You agree that LAU may, without changing the content, translate the submission to any medium or format for the purpose of preservation. You also agree that LAU may keep more than one copy of this submission for purposes of security, backup and preservation. You represent that the submission is your original work, and that you have the right to grant the rights contained in this license. You also represent that your submission does not, to the best of your knowledge, infringe upon anyone's copyright. If the submission contains material for which you do not hold copyright, you represent that you have obtained the unrestricted permission of the copyright owner to grant LAU the rights required by this license, and that such third-party owned material is clearly identified and acknowledged within the text or content of the submission. IF THE SUBMISSION IS BASED UPON WORK THAT HAS BEEN SPONSORED OR SUPPORTED BY AN AGENCY OR ORGANIZATION OTHER THAN LAU, YOU REPRESENT THAT YOU HAVE FULFILLED ANY RIGHT OF REVIEW OR OTHER OBLIGATIONS REQUIRED BY SUCH CONTRACT OR AGREEMENT. LAU will clearly identify your name(s) as the author(s) or owner(s) of the submission, and will not make any alteration, other than as allowed by this license, to your submission.

Name: Charbel Elkhoury

Signature: [redacted]

Date: 26/07/2020

LAU
الجامعة اللبنانية الأميركية
Lebanese American University

PLAGIARISM POLICY COMPLIANCE STATEMENT

I certify that:

1. I have read and understood LAU's Plagiarism Policy.
2. I understand that failure to comply with this Policy can lead to academic and disciplinary actions against me.
3. This work is substantially my own, and to the extent that any part of this work is not my own I have indicated that by acknowledging its sources.

Name: Charbel Elkhoury

Signature: 

Date: 26/07/2022

ACKNOWLEDGEMENT

I would like to deeply thank my advisor, Dr. Moustapha Harb, whom continuous guidance and support made the completion of my master's degree possible. Also, I would like to extend my gratitude to my thesis committee, Dr. Mahmoud Wazne, and Dr. Sima Tokajian. Finally, a thank for my colleagues who contributed to this work, Miss Christelle BouNehme Sawaya, Miss Lama Ramadan, and Miss Reem Deeb.

Assessing intracellular and extracellular antibiotic resistance gene proliferation during the treatment of municipal wastewater by an anaerobic membrane bioreactor

Charbel Elkhoury

Abstract

As water scarcity is emerging as a great threat nowadays, water reuse is a critical component of the solution. Among many tested wastewater treatment technologies, anaerobic membrane bioreactors (AnMBRs) have proven their unique treatment capabilities for producing high quality effluents and recovering energy. The removal of emerging microbial contaminants from wastewater, such as antibiotic resistance genes (ARGs) and uncultivable pathogens, has not been extensively studied for AnMBRs during treatment of real (non-synthetic) municipal wastewaters. Hence, this research aimed to assess the ability of AnMBRs to achieve removal of both extracellular and intracellular ARGs (eARGs and iARGs, respectively), as the two can proliferate through vastly different means in wastewater treatment environments. The experiment consisted of a lab-scale AnMBR treating a local wastewater source under various conditions. The experiment was designed to compare ultrafiltration (UF) and microfiltration (MF) membrane effluent profiles at different transmembrane flow rates and different membrane fouling levels. The phases of operation included: a transition from synthetic to real wastewater, stepwise increases in transmembrane flux, and post membrane replacement to assess the effect of membrane fouling. It was observed that higher effluent abundances of iARGs were related to higher transmembrane flux rates while eARGs were not directly affected by flux. eARGs were also more abundant overall in the effluent, which appeared to be a direct result of corresponding influent wastewater profiles. Moreover, it was found that the fouled UF membrane was more effective at removing certain genes (such as *sul1* and *intI1*) as compared to an unfouled UF membrane. This study provides new insights regarding ARG dynamics in AnMBR effluents by elucidating the effect of operating different membrane types, maintaining membrane biofilms, and controling transmembrane flux rates on the nature of ARGs released into the permeate.

Keywords: Antibiotic resistant genes, Fouling, Transmembrane Pressure, Intracellular, Extracellular, AnMBR, Transmembrane Flux, Ultrafiltration, Microfiltration.

TABLE OF CONTENTS

Chapter **Page**

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure Page

List of Abbreviations

AD	anaerobic digestion
kDa	kilo Dalton
AeMBR	aerobic membrane bioreactor
AnMBR	anaerobic membrane bioreactor
ARG	antibiotic resistance gene
COD	chemical oxygen demand
CSTR	continuously stirred tank reactor
eARG	extracellular antibiotic resistant gene
iARG	intracellular antibiotic resistant gene
HRT	hydraulic retention time
LC-MS	liquid chromatography mass spectrometry
LRV	log removal value
MBR	membrane bioreactor
MF	microfiltration
UF	ultrafiltration
MW	molecular weight
NCBI	National Center for Biotechnology Information
CDC	Centers for diseases control and prevention
OTU	operational taxonomic unit
PVDF	polyvinylidene fluoride
qPCR	quantitative polymerase chain reaction
SRT	solids retention time
QMRA	Quantitative Microbial Risk Assessment
TMP	transmembrane pressure
WWTPs	wastewater treatment plants

Chapter One

Introduction

1.1. Anaerobic membrane bioreactor: A promising technology

As the world population increases and technology progresses, the conception of wastewater reuse has been recently introduced as an important engineered solution. For that purpose, many emerging technologies were adopted, perhaps the most common ones being activated sludge-based processes for secondary wastewater treatment such as the membrane bioreactor (MBR). However, as such aerobic treatments require extensive energy use and cost for aeration, which in its turn is becoming a scarce resource, moves toward anaerobic treatments methods are being studied lately (Baek and Pagilla 2006).

MBRs are emerging wastewater treatment technologies with many advantages, among which is the combination of biological and physical treatment of wastewater, using sludge and membranes filtrations. The high sludge retention times allow for the development and growth of sludge microbiomes for appropriate wastewater types by reducing the washout of those organisms, which enhances the efficiency of the treatment. Moreover, MBRs allow for the separation of hydraulic retention time (HRT) and sludge retention time (SRT), permitting to process a higher amount of influent wastewater while simultaneously using smaller reactor volumes by shortening the HRT and increasing the SRT (Dagnew and Parker 2021). All of the above mentioned benefits (along with many others) distinguish the MBRs from conventional wastewater treatment systems. Furthermore, MBRs can be divided into aerobic systems and

anaerobic systems, however, the anaerobic variants have shown better energy recovery and less energy consumption due to their ability to produce methane (Smith, Stadler et al. 2014).

The anaerobic membrane bioreactor (AnMBR) is a newly emerging technology that is based on anaerobic digestion (AD), which has attracted many researchers in recent years as has shown its efficiency in producing good quality effluent while minimizing energy cost and maximizing energy recovery. The biological part of the AnMBR encompasses acidogenic, acetogenic and hydrolytic bacteria which, in combination with methanogenic archaea, are able to decompose organic compounds into methane and carbon dioxide (CO_2), mitigating at the same time their negative effect on the environment (Inaba, Su et al. 2020). Those, among many other sets of bacteria present in the AnMBR, have proven their efficiency in treating various types of wastewaters that include highly polluted municipal wastewater, food waste, and industrial wastewater.

AnMBR units are composed of two main constituents. The first, which is responsible for the biological treatment, is commonly operated as a continuously stirred tank reactor (CSTR). The second consists of filtration membranes that can be of different sizes and can be mounted in two configurations: either submerged in the CSTR or as external cross flow membranes. Each of these mounting configurations for the membranes can be advantageous, for instance, submerged membranes require less energy as they do not require flow recirculation, however cross flow membranes permit easier operation as they are easier to be reached. Membranes used in AnMBRs are usually hollow fiber or flat sheet membranes. Other parameters that must be configured while operating AnMBRs are the organic loading rate, HRT, and transmembrane flow rate that play a key role in the performance of such systems. Moreover, for the biological treatment process to be optimized, many parameters need to be considered. Those include pH,

reactor temperature, loading rate and the seed sludge composition (Kanafin, Kanafina et al. 2021). First, for digestion to take place, a pH of 6.5-7.5 must be maintained in the digestor, otherwise the activity of the microbial community can be inhibited. A temperature of 35 °C for mesophilic activity and 55 °C for thermophilic activity were found to be optimal (Lew, Tarre et al. 2009). Since deviations in the previously mentioned parameters can severely alternate the activity and nature of the microbial community in the digestor, many studies were developed to find the optimal operating conditions of AnMBRs in order to improve their treatment efficiency and effluent quality.

In recent years, AnMBRs have proven to be a promising technology for delivering consistent water effluent that can be reused or discharged into the environment. Perhaps one of the most suitable reuse application is wastewater reuse for irrigation, as AnMBR effluent is known to be rich nitrogen and phosphorus (Harb and Hong 2017). However, a direct concern for this application is the existence of microbial pollutants in effluent such as pathogens, antibiotics and antibiotic resistant genes (ARGs), whose presence can be a main inhibitor of water reuse in various applications as they pose serious threats (Harb and Hong 2017). Yet, as water scarcity is becoming more and more serious, significant work is still required to correctly understand the capability of AnMBRs towards the removal of microbial contaminants. Among these microbial contaminants, ARGs are among the most concerning due to their severe long-term implications.

1.2. Background: Antibiotic resistance genes

The inevitable use of antibiotics throughout the years has contributed to the expansion in ubiquity of antibiotic resistant genes. Antibiotic resistance is when microorganisms develop the ability to become immune to the effects of the drugs that are designed to kill them. According to the CDC, antibiotic resistance is responsible for 48,000 deaths yearly in the US only, which

make it an emerging threat that must be dealt with. Moreover, not only can bacteria become resistant to antibiotics, but they have the ability to transfer its resistance to other bacterial types in a process called horizontal gene transfer (HGT) (Van Hoek, Mevius et al. 2011). The transfer of resistance between bacteria can also happen from dead bacteria to live bacteria or from mobile genetic elements floating freely in liquid suspension in the environment (Van Hoek, Mevius et al. 2011). In addition, it is known that non-pathogenic bacteria can be a source of antibiotic resistance that is transferrable to pathogenic ones (Miller, Novak et al. 2016).

The overuse of pharmaceutical products and the large use of antibiotics in various agricultural industries (e.g., the poultry processing industry) have led to the spread of antibiotic resistance genes to almost all wastewater types, further magnifying the challenge of water reuse. Moreover, wastewater treatment plants constitute a pool for antibiotic resistance resulting in high frequencies of horizontal gene transfer, therefore elevating the risk in receiving environments (Schlüter, Szczepanowski et al. 2007, LaPara, Burch et al. 2011). In addition, treatment plant sludge, rich in nutrients and microorganisms that can host antibiotic resistance genes, has been found to be linked with the persistence of those genes in treatment plant systems (Miller, Novak et al. 2016).

AnMBRs, with their high sludge retention times can constitute a reservoir suitable for HGT conditions and the long exposure of sludge to antibiotics can lead to an increase in the degree of resistance of existing bacteria by applying selective pressure. Yet still, the contribution of AnMBRs to this proliferation of ARGs remains largely understudied (Zarei-Baygi, Harb et al. 2019). Moreover, little is still known about the capability of AnMBRs in the removal of such contaminants from different types of wastewaters (Harb and Hong 2017).

ARGs can exist in both extracellular (eARGs) and intracellular (iARGs) form, with each having unique properties that affect transfer rates and mechanism of actions that can impact the abundance of each in AnMBR permeates (Zarei-Baygi, Wang et al. 2020). Therefore, understanding AnMBR contribution to the removal of both aspects of those contaminants is a must. Among different types of wastewaters, municipal wastewaters are commonly found to be rich in ARGs and pathogens, as they receive wastewaters from different facilities that are rich such contaminants (such as hospital wastewaters) (Osińska, Korzeniewska et al. 2017).

As the treatment of ARGs and pathogens in real municipal wastewater (non-synthetic) remains an understudied topic, targeted research is still needed to understand the efficiency of such treatment systems in contaminants removal, specifically to the extracellular and intracellular level. No previous work has been done in a targeted system operation-based manner to understand the behaviors of extracellular and intracellular ARGs in AnMBRs treating real municipal wastewater. Also, the contribution of the effect of having filtration membranes with different pore sizes accompanying AnMBRs in the removal of such contaminants and the effect of membrane fouling are still unknown.

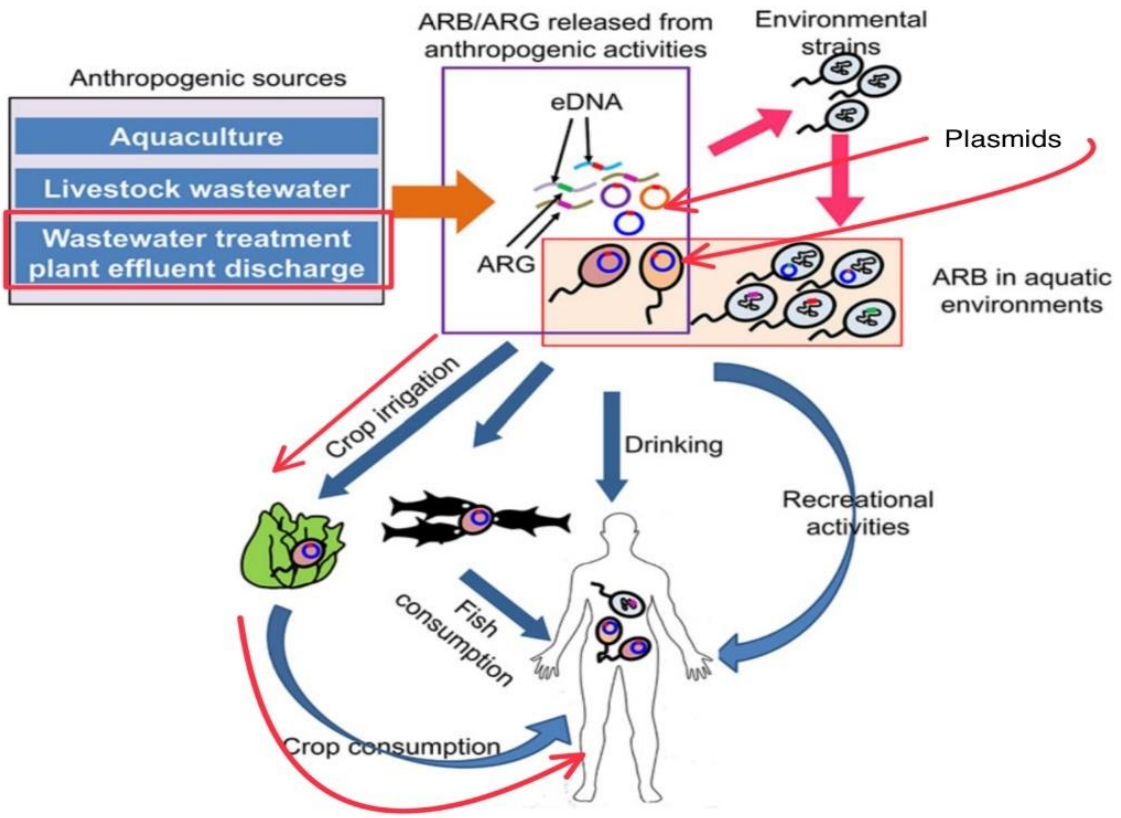

Figure 1 Schematic representation of ARGs dynamics in the environment adapted from (Amarasiri, Sano et al. 2020)

Chapter Two

Literature Review

2.1. Water scarcity and reuse concerns

Wastewater treatment's initial purpose was to permit the safe discharge of used waters in a way that abides to federal regulations so as to protect the environmental security and human health. However, nowadays drought periods are becoming more severe and climate change is becoming a serious threat, as is the need for food security. As such, the purpose of wastewater treatment systems has shifted towards providing solutions that permit safe wastewater reuse for various applications instead of just discharging them into water streams. To face this issue, many wastewater reuse schemes are starting to be implemented, such as industrial reuses, non-potable urban reuses, and others (LaPara, Burch et al. 2011).

Among other wastewater reuse applications is wastewater reuse for agriculture irrigation, which currently helps in irrigating 31% of crops in California–175.1 million m^3 of water per year. Still, only 27 US states have regulations allowing for irrigation of food crops (Ritter 2021), with similar inconsistency in regulations in Europe. This application, however, provides many advantages that include socio-economic benefits, less need for fertilizers, and preservation of waterways (Fatta-Kassinos, Kalavrouziotis et al. 2011). However, in contrast to its advantages, this application also poses serious threats on the environment and human health. Such threats lie mainly in the presence of pathogens and other associated microbial contaminants in the effluent of treatment plants such as ARGs.

Thus, many risk models such as quantitative microbial risk analysis (QMRA), Monte Carlo analysis and others were developed to evaluate the risk of wastewater reuse for crop irrigation, or further to be able to quantify this risk and evaluate the danger it imposes on human health within set thresholds. For instance, the QMRA method, analyzes the risks imposed by crop consumption that are irrigated by treated wastewater, with the consideration of different scenarios and factors. The output of this quantification method should be below a set threshold that reflects the annual risk of infection specified by the world health organization (Hamilton, Stagnitti et al. 2006). QMRA has been widely used over the years by scientist and was found to be effective for this risk quantification purpose. Yet, this method does not accommodate for increased risk due to the potential for acquisition of antibiotic resistance in any of its factors, components, or models, hence no present method is available for the quantification of the added dangers of ARGs when it comes to wastewater reuse for irrigation. Moreover, the ability of ARGs to proliferate through HGT in the environment and the little information available on the frequency of this transfer with respect to different pathogenic and non-pathogenic hotspots in reuse practices is still unknown and thus difficult to be accounted for (Hamilton, Stagnitti et al. 2006). This makes the dangers associated with wastewater reuse in crops irrigation a bigger threat. Hence, the ability of many wastewater treatment systems to treat ARGs from different types of wastewaters has been a topic of interest in recent years.

Among those systems, AnMBRs are being widely examined as their nutrient rich effluent, high energy recovery potential and good treatment efficiency makes them good candidates for water reuse in irrigation. However, little is still known regarding the ability of those systems in the treatment of microbial contaminants from wastewater, and therefore the risks imposed by the reuse of AnMBRs effluent in agriculture irrigation.

2.2. Pathogens and ARGs presence in wastewaters

The overuse of antibiotics in both domestic and agricultural settings combined with the role that they play in applying natural selective pressure have led to the proliferation of antibiotic resistance in almost all type of wastewater. Their presence is unpredictable as they can be found in both natural and engineered environments (Viswanathan 2014). Thus, the study of ARG and pathogen presence in wastewater treatment plants (WWTPs) treating different types of wastewaters has become a topic of great interest over the past several decades.

Among different type of wastewaters, municipal wastewater and agro-industrial wastewaters were found to be the richest in ARGs and pathogens. Hospitals, butchers, household pharmaceutical compounds and many other facilities contribute to the richness of ARGs and pathogens in municipal wastewater. As for agro-industrial wastewater with livestock processes, the extensive use of antibiotics and other pharmaceutical compounds necessary for those facilities is the main driver for the richness of such wastewater with these microbial contaminants (Czekalski, Gascón Díez et al. 2014).

2.2.1. Antibiotic resistance genes: A growing threat

Resistance to a wide spectrum of antibiotics that include sulfonamides, methicillin, carbapenem, beta-lactams, and other classes of antibiotics has now become relatively ubiquitous. Furthermore, some genes are also associated with multidrug resistance, which allows its host to be resistant to different antibiotic classes simultaneously (Dever and Dermody 1991). Different mechanisms of antibiotic resistance are currently known which include: the development of new cell processes that avoid using the target drug, altering the enzymes responsible for the drug consumption, modification of entryways thus blocking drug entry, changing antibiotic target site so it can no longer attach, and finally modifying cell pumps to pump out the antibiotic (Reygaert 2018).

Such ARGs exist abundantly in municipal wastewater worldwide and it was found that genes providing resistance to all antibiotic classes previously mentioned, such as *sul1* and *sul2* genes, *tetO* genes, *ermB* genes and others that are affiliated with HGT (e.g., *intl1* genes), are commonly detected (Rafraf, Lekunberri et al. 2016, Neudorf, Huang et al. 2017). Moreover, for industrial wastewaters, such as poultry slaughterhouse wastewater, different ARGs were proven to be existent in alarming concentrations as well. Those include *blaTEM*, *mcr1*, *sul1*, *ermB* and others (Savin, Alexander et al. 2021).

Another aspect that can differentiate the mechanism of aquisition of ARGs and therefore their effects is the form in which they exist, i.e., whether as extracellular (eARGs) or intracellular (iARGs) genes. iARGs are ARGs that exist inside bacteria (either on plasmids or the genome) making it resistant to antibiotics, whereas eARGs are resistance genes floating freely as genes outside of cells (either as fragments or on plasmids). eARGs have an increased ability of facilitating HGT through transformation, whereas iARGs can also outgo HGT through conjugation (Sui, Chen et al. 2019). Conjugation is the dissemination of genetic material from cell to cell through direct contact, however transduction of iARGs can also occur through transfer by bacteriophages (Liu, Qu et al. 2018). Horizontal gene transfer frequency varies based on the donor bacteria, the recipient bacteria, types of mobile genetic elements (MGEs) present, the resistance gene itself, and the transfer method (Silva, Loreto et al. 2004). Yet, little is known about the frequency of ARGs transfer in municipal and industrial wastewaters, as well as in the environment. Considering that eARGs undergo HGT via natural transformation (which is the third mechanism of HGT), the frequency of this transformation occurs similarly to iARG transfer mechanisms in some environments such as seawaters (Dong, 2019). This makes the study of eARG presence in effluents an equally important matter, as they contribute extensively to the

proliferation of antibiotic resistance in the environment (Zarei-Baygi and Smith 2021). Moreover, the effect of antibiotics on the presence and manifestation of those ARGs is greatly dependent on their form of existence, as ARGs persist after host cell death or antibiotic removal due to their "easy to get, hard to lose" characteristic (Dong, Wang et al. 2019).

2.2.2. Pathogens in wastewaters: Presence and relation to ARG proliferation

One of the reasons for the proliferation of ARGs is the gene transfer between bacteria in a way that those bacteria can serve as vectors for antibiotic resistance and, worse, transfer them to pathogenic bacteria. This can pose a serious threat on environmental security and human health as pathogens with acquired antibiotic resistance are twice as lethal, and have more severe effects on human health due to their increased virulence (Berendonk, Manaia et al. 2015, Sharma, Johnson et al. 2016, Cheng, Ngo et al. 2018). Those pathogens include but are not limited to *Klebsiella*, *Escherichia*, *Pseudomonas*, *Salmonella* spp. and others, which are present in alarming concentrations in municipal wastewater (Shannon, Lee et al. 2007), with *Pseudomonas, Staphylococcus, Salmonella* spp. and others being found in slaughterhouse wastewaters (Meiramkulova, Temirbekova et al. 2021). For instance, it was found that pathogens are abundant in municipal wastewaters and their associated treatment systems, with concentrations for genomic DNA as high as 10^8 copies/ug in the sludge of municipal wastewater treatment plants (Lee, Shannon et al. 2006). Furthermore, not only do the previously mentioned pathogens constitute a huge risk on their own by posing serious threats to human health, but they are also known for their high potential for HGT, magnifying their effects and dangers and helping in the proliferation of such genes (Amarasiri, Sano et al. 2020). It was found that conjugation of ARGs between different bacteria contributes significantly to the proliferation of those contaminants in ecosystems, especially in soil and water environments such as agricultural fields (Von

Wintersdorff, Penders et al. 2016). Moreover, it was observed that almost all antibiotic resistant pathogens are capable of natural transformation and DNA uptake in clinical environments, which increases the dangers of antibiotic resistance spread (especially given that those pathogens are widely present in municipal wastewaters) (Shannon, Lee et al. 2007, Lerminiaux and Cameron 2019). Moreover, a novel study by Zarei-Baygi et al., 2020, found a strong correlation between some Operational Taxonomic Units (OTUs) and ARGs, which may indicate that some OTUs can serve as hosts for ARGs and affect in their spread and increased dangers in wastewater treatment systems such as the AnMBR.

The co-presence of such ARGs and pathogens in municipal and slaughterhouse wastewaters in high concentrations emphasizes the importance of studying new treatment systems' effectiveness in their removal (such as the AnMBR), as the form in which they exist in such newly developing treatment systems is still an understudied topic.

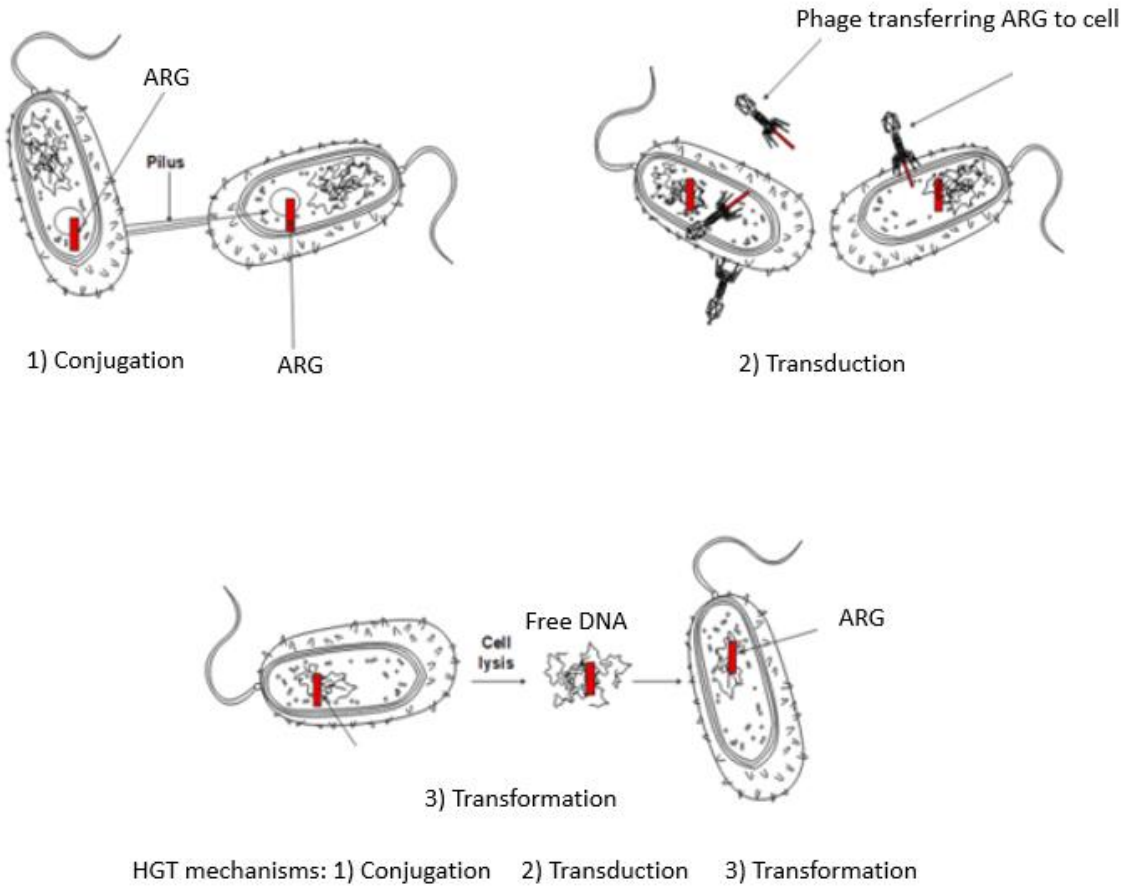

Figure 2 Horizontal gene transfer mechanisms adapted from (Aziz, Sengar et al. 2022)

2.3. AnMBRs and microbial contaminants: Removal efficiency and presence

Given that ARGs and pathogens are present abundantly in a diverse range of wastewater types, treatment efficiency of wastewater treatment plants with different treatment techniques in the removal of such microbial contaminants has become an area of investigation. The effectiveness of secondary and tertiary treatments in conventional WWTPs regarding the removal of ARGs and pathogens have been thoroughly examined. However, it was found that even advanced treatment systems such as UV tertiary treatment can only slightly reduce the concentration of ARGs in wastewater (as low as 0.5 log reduction) (Sui, Chen et al. 2019).

MBR systems gained recent attention for wastewater treatment, and their study showed robust ARGs and pathogens removal, along with other notable advantages such as cost efficiency. This efficiency is achieved by the fact that MBRs combine biodegradation/biosorption with membrane filtration. It was found that multiple types of MBRs such as aerobic MBRs, anoxic/aerobic MBR and others can achieve ARGs removal to up to 4 log removal values (LRV) (Wang and Chen 2022). Moreover, not only were those systems found to be more effective than conventional systems in terms of effluent concentrations, but also carry lower concentrations of ARGs in their biomass. For example, the concentrations of *ermB, sul1*, *intl1*, *tetO* and others were found to be lower in MBR sludge compared to conventional plants (Le, Ng et al. 2018).

AnMBRs have proved to be consistently efficient in the removal of ARGs with LRVs ranging from 3.3 to 3.6 (Cheng and Hong 2017, Kappell, Kimbell et al. 2018, Wang and Chen 2022). This makes those systems particularly unique for wastewater treatment, as not only are they efficient in the removal of emerging microbial contaminants but can lower operation costs by methane recovery from the reactor and the effluent as well (Harb, Wei et al. 2016).

The study of antibiotic resistance in AnMBRs therefore started to be examined by many researchers for different type of wastewaters, as it is still an understudied area. In studies that examined ARG profiles in AnMBR system sludge biomass and effluent, it was found that the sludge biomass component was rich in different ARGs, namely *ermF, ermB, sul1*, *tetO* and others. It was found as well that the effluent of this system carried different ARG profiles (Zarei-Baygi, Harb et al. 2019, Zarei-Baygi, Harb et al. 2020, Zarei-Baygi, Wang et al. 2020). Moreover, AnMBRs were found to be effective in the removal of ARGs from co-treated influents. For instance, in their study, Lou et al. (2020) found that not only was an AnMBR efficient in the removal of ARGs while co-treating livestock manure and domestic wastewater,

but the complex mix led to higher energy recovery, and effluent rich in nutrients for irrigation reuse.

In addition, the combination of AnMBRs with other treatment techniques was proven to be beneficial in ARG removal. For instance, UV/H_2O_2 treatment of AnMBR effluent was found to be effective in the supplemental removal of such contaminants (Augsburger, Zaouri et al. 2021). Further, adding an electrochemical system to an AnMBR was as well effective in the removal of various emerging contaminants including ARGs (Li, Yuan et al. 2021).

Moreover, studies have proven that AnMBRs are efficient in the treatment of not only ARGs but pathogens as well, reducing the chances of ARGs proliferation in the effluent through HGT as previously discussed. For instance, *E. coli, Enterococci*, *Clostridium perfringens*, and coliphages were successfully removed by an AnMBR system at up to 6.4 LRVs (Wong, Xagoraraki et al. 2009, Harb and Hong 2017). However, other studies found that AnMBR effluent can still carry certain pathogens that might facilitate ARG proliferation (Harb and Hong 2017, Zarei-Baygi, Harb et al. 2020)

AnMBR efficiency in ARG removal from different types of wastewaters is still a developing topic. However, as it is found to be promising based on results so far, the study of such systems in the treatment of real municipal wastewater is an important understudied aspect, especially to the level of differentiating between extracellular and intracellular ARGs.

2.4. Effect of membrane pore sizes and fouling on ARG removal in AnMBRs

Filtration membranes play a key role alongside the biological treatment in AnMBRs, not only because they provide physical-based separation because of their small pore sizes, but more they can also provide biological treatment when biofilm development occurs.

Filtration membrane pore sizes cover a wide range from microfiltration to reverse osmosis and the operating energy requirement varies drastically between the sizes, with lower sizes requiring more energy in terms of pressure differential. Thus, it is crucial to operate AnMBR systems with the most convenient membrane size to balance between the desired effluent quality and energy balance. In their study, Jiayuan et al. (2020), found that a 0.05 µm membrane and 0.4 µm membrane treating municipal wastewater achieved similar performance in terms of BOD/COD removal and suspended solids removal while having the same energy recovery yield. However this study did not investigate the effect of membrane sizes on ARGs in the effluent. Another study comparing the performance of membranes with different pore sizes in the removal of ARGs (generally) found that an ultrafiltration membrane with pore size of 10 kDa achieved significantly better LRVs than a microfiltration membrane. This study also determined that the molecular weight of ARGs plays as a key role in the filtration process, as smaller ARGs passage was not completely excluded by a 1 kDa membrane. This not only emphasizes the importance of membrane pore sizes in ARG removal considerations, but the anticipated sizes of the target genes as well (Breazeal, Novak et al. 2013). However, the previous study examined the membranes processes in series (as a sequential filtration) and was only limited to the physical separation process. Thus, no studies previously compared ARGs and pathogen removal efficiency of AnMBRs combined with two filtration membranes of different sizes in parallel.

According to previous studies, biofilms are EPS and SMP layers that form on the membrane (cake layers) or are adsorbed into the membrane pores (pore blockage) (Vrouwenvelder, Kruithof et al. 2010). The study of those fouling layers had gained researchers' attention as they greatly contribute to the AnMBR performance by encompassing microbial communities with unique characteristics such as methanogenic, syntrophic and fermentative groups (Ozgun, Dereli

et al. 2013, Cheng, Cheng et al. 2019, Guo, Cheng et al. 2020). Those groups possess unique capabilities such as biotransformation of OMPs, degradation of antibiotics, and methane production (Gonzalez-Gil, Carballa et al. 2017, Gonzalez-Gil, Mauricio-Iglesias et al. 2018, BouNehme Sawaya and Harb 2021). Moreover, those biofilm layers have been found to be rich in potential pathogens such as *Acinetobacter, Pseudomonas*, and others under certain operating conditions, which can also affect ARG distributions in the effluent (Sawaya et al., in review). Moreover, it has been found that the removal of eARGs is strongly related to the EPS and SMP content of the membrane (Robles, Serralta et al. 2021).

Thus, another aspect that is understudied and must be focused on is the effect that biofilms and membrane fouling have on eARG and iARG profiles in AnMBRs effluent. For instance, it was found that low fouling rates were the best for ARGs removal in AnMBRs for iARGs, while highly fouled membranes are more efficient in the removal of eARGs (Cheng and Hong 2017, Zarei-Baygi, Wang et al. 2020) , which could be a matter of size exclusion in terms of kDa but on a biofilm matrix-basis. Still, those studies examined the effect of fouling for AnMBRs treating synthetic wastewater and not real wastewater conditions. Moreover, it is worthy to note that HGT mechanisms in AnMBR membrane biofilms is still an understudied topic.

Chapter Three

Aim and Objectives

3.1. Research Aim

The current study aim was to examine the proliferation of iARGs and eARGs in an AnMBR equipped with one microfiltration membrane and one ultrafiltration membrane after the transition from synthetic influent to real municipal wastewater, while also investigating the effect of modifying the operating conditions on this proliferation. Overall, the study aimed at understanding the efficiency of the AnMBR in the removal of eARGs and iARGs from municipal wastewater, which can provide valuable insights about the potential for AnMBR effluent reuse in irrigation. Moreover, the effect of poultry slaughterhouse wastewater introduction on the ARG profiles of an AnMBR was also examined.

3.1. Research Objectives

1. Determine antibiotic resistance profile in different wastewaters:

1.1. ARGs profile in poultry slaughterhouse wastewater

1.2. ARGs profile in low strength municipal wastewater

1.3. ARGs profile in high strength municipal wastewater

2. Evaluating the effect of transitioning from synthetic influent to real wastewater influents on ARG profiles in the AnMBR.

3. Evaluate the performance of AnMBR in overall ARGs removal from municipal wastewater

4. Examine the need of using filtration membranes of different sizes:

4.1. Performance of microfiltration membrane in ARGs removal

4.2. Performance of ultrafiltration membrane in ARGs removal

5. Determine the effect of biofilms and flow rate variation on eARGs and iARGs profiles in the effluent

6. Identify the best strategy for ARGs removal

3.3. Scope of Work

The experiment consisted of an AnMBR system for which influent was transitioned from synthetic to real municipal wastewater retrieved from several municipal wastewater treatment plants. The system was operated at mesophilic conditions with a temperature of 35 °C throughout the experiment. One ultrafiltration and two microfiltration membranes were operated in parallel at a constant flow rate initially, which was then increased throughout the experiment to allow biofilm development on the membranes and examine the effect of such increase on ARG profiles. After membrane fouling was reached, the ultrafiltration and one microfiltration membranes were harvested and were replaced by two new ones of the same properties, as the second fouled microfiltration membrane was kept for comparison purposes. Throughout the experiment, effluents samples were taken from the effluent of each membrane weekly or prior to each flow rate change and influent samples were taken for each time the influent was changed. Moreover, chemical oxygen demand (COD) was measured regularly. Effluent methane concentrations were also measured and biomass samples for microbial characterization were taken. Moreover, another system with the same operating conditions treating synthetic wastewater was ran, in which a transition from synthetic influent to poultry slaughterhouse

influent was performed so as to examine the effect of such a change on the ARG profiles of the AnMBR system.

Chapter Four

Methodology

4.1. Anaerobic Membrane Bioreactor Configuration

The anaerobic membrane bioreactor (AnMBR) consisted of a lab scale continuously stirred tank reactor (CSTR), 3-L working volume (Chemglass Life Science, USA), mixed using an internal impeller at 200 rpm. Mesophilic conditions were maintained by a water-jacket with flowing water at 35 °C. The experiment consisted of two phases, the first to allow the membranes to foul by increasing the flow rate, and the second in which two new membranes and an existent one were operated at a constant flow rate. Three external cross-flow flat sheet membrane modules were operated in parallel while ensuring that all three membranes had the same cross-flow conditions. The three membranes used were made of polyvinylidene difluoride (PVDF) and consisted of two microfiltration membranes with a pore size of 0.2 µm and one ultrafiltration membrane with a pore size of 0.05 µm (Microdyn Nadir, Germany) with an effective area of 0.057 m^2 each. Backwash for 30 minutes was applied daily for each membrane at three times the operating flow rate, by sending distilled water from the permeate side to the opposite side of filtration to prevent membrane pores blockage. Recirculation of the biogas was applied on the membrane surfaces that were relaxed for 60 seconds every 1 hour. The sludge that seeded the reactor were retrieved from an anaerobic digestor in Lebanon and was fed synthetic wastewater before transitioning to real municipal wastewater obtained from two major wastewater treatment plants in Lebanon, after the screening process only, at the beginning of the experiment. The organic loading rate was maintained between 0.9-1.2 gCOD/L-d throughout the experiment. pH

was kept at near 7 and the sludge retention time (SRT) was 360 days. The flux rates were controlled by setting different effluent pumping rates on a peristaltic standard digital pump (Masterflex, United States). External pressure gauges were used to monitor the transmembrane pressure (TMP).

Another AnMBR system with the same operating conditions was ran along with the first one and was fed synthetic wastewater to ensure sludge growth and maintenance. One MF membrane with the same type and pore size was installed, and a transition from synthetic to chicken slaughter waste influent was made at the start of the experiment.

4.2. Water quality testing and sampling

AnMBR physical and chemical properties were examined and included the chemical oxygen demand (COD), volatile and suspended solids (TSS/VSS), and volatile fatty acids (VFAs), all following standard procedures. The COD of the influent and permeates was measured twice a week by a Hach DR3900 Spectrophotometer following USEPA Reactor Digestion Method and colorimetric determination. In a brief, 2 mL of the sample were pipetted in vials that contain dichromate and put in a digestor at 150 °C for 2 hours. Then the samples were measured at 600 nm after blanking the equipment, and the COD removal was determined as:

$$(CODInfluent - CODEffluent)/CODInfluent \times 100$$

with all the corresponding CODs in mg/L.

Volatile fatty acids (VFAs), which encompass acetate, propionate, and butyrate concentrations in the effluents, were tested twice weekly. Samples were taken from each membrane permeate then filtered using 0.2 µm nylon syringe filters prior to being injected into the 882 Compact IC plus ion chromatography machine equipped with a conductivity detector and 858 Professional Sample

Processor (Metrohm AG, Switzerland). Standards curves for acetate, propionate, and butyrate were in the ranges of 5 mg/L, 25 mg/L, 50 mg/L, 100 mg/L, 250 mg/L, and 500 mg/L, respectively.

4.3. Biogas and effluent methane testing

The daily biogas produced by the reactor was captured in 1-L Tedlar bags from which 300 mL was collected for sampling, and the remaining volume emptied while recording the volume. The quantification of the possible present gases was performed using gas chromatography technology (Agilent 7890B) equipped with a thermal conductivity unit (GC-TCD), and oven temperature set at 90°C. As for the detector, a temperature of 250°C was adopted. The targeted gases were CH_4, CO_2, and N_2, for which percentages were determined by the instrument then methane content was normalized to CH_4 and CO_2 and thereafter the actual methane volume was calculated (Appendix A). The theoretical methane volume was then obtained using the formulas in Appendix A.

As for the determination of dissolved methane in the effluent, weekly samples of a volume of 30 mL were taken using a syringe, which was added to a 250 mL flask pre-filled with Nitrogen. Thereafter the sample was shaken for 1 minute, heated for 10 minutes, and shaken again briefly in order to heat the methane to the gas phase. The sample was then run on the GC-TCD machine. In order to evaluate the dissolved methane content, a ratio known by the oversaturation ratio was calculated.

4.4. Effluent sampling for ARGs quantification

For effluent sampling and ARGs quantification, permeate collection flasks were sterilized a night prior to the sampling day, and a permeate volume of at least 500 mL was ensured to be in the

flask prior to the sample collection for it to be representative. Prior to sample collection, the flasks were thoroughly mixed to ensure sample homogeneity, then 120 mL of each permeate of the three membranes were filtered using 0.45 um membrane filters (Millipore, USA) that were then stored at – 20°C for intracellular DNA extraction. As for extracellular DNA extraction, permeate of the filtration was then spiked using 2 µL of pGEM 3-z vector (Promega, USA) to later calculate the recovery efficiency. After, isopropanol was added to the spiked permeate at a 1:1 ratio and then the mix was left at – 20°C overnight for pellets precipitation to occur. Thereafter, the mix was centrifuged at 14,500 rpm for 30 minutes to cause the eDNA to pellet, and pellets were washed with 70% ethanol to remove any residual isopropanol. Following was the centrifugation of the pellets performed at the same speed for 20 minutes, and the supernatant was discarded. Pellets were then allowed to air dry for 5 minutes so that remaining traces of ethanol evaporate and were stored at – 20°C until the extraction. This method was a modified method based on the Zarei-baygi et. Al (2020) adopted method. The same method was applied to extract extracellular DNA from the reactor sludge, with initial centrifugation at 6,000 rpm for 10 minutes for filterability of the supernatant. As for intracellular sludge samples, 2 mL of sludge were centrifuged at 12,000 rpm for 10 minutes, the supernatant was discarded and pellets were stored at – 20°C. All glassware used was autoclaved at 120°C for 30 minutes, and thereafter baked at 400°C for an hour to ensure that no volatile particles are present.

4.5. Methods optimization for ARGs quantification

DNA extractions on the stored samples were performed using DNeasy PowerSoil Kit (Qiagen, USA) according to the manufacturer's latest protocol. Thereafter, the concentration and quality of the extracted DNA was tested on a Nanodrop ND 1000 spectrophotometer Version 3.3.0 with

recent calibration. Then, extracted samples were stored at -20°C until the quantitative PCR (qPCR) was performed.

Genes that confer resistance to different classes of antibiotics and are normally found abundantly in wastewaters were targeted. Those included: Sulfonamides (*sul1* and *sul2*), β-lactamase (*ampC* and *blaTEM*), tetracycline (*tetC*, *tetQ*, *tetW*, *tetA*), colistin (*mcr1*), and erythromycin (*ereA*, *ermF*). Municipal wastewater samples collected from a large wastewater treatment plant were used to amplify the corresponding genes by PCR in order to prepare the qPCR standards.

In order to optimize qPCR amplification efficiency (which should be above 90%), many procedures were tested, and the following procedure and mixes were found to be optimal. For the PCR reaction, the appropriate primers and thermocycling conditions were used for each gene as shown in Table 1 with a mix consisting of 4 uL of 5x FIREPol master mix (Solis BioDyne, USA), 1 uL of each reverse and forward primer with a concentration of 5 uM, 2 uL of DNA template, and 12 uL of molecular grade water to have a 20 uL reaction. Thereafter, 2 uL of the PCR product was used to run gel electrophoresis on a 1.5% agarose gel, and bands were visualized using a ChemiDoc Touch Imaging System (Bio-Rad Laboratories, USA) in order to confirm based on the base-pair length that the targeted gene is the one that was amplified. After confirmation, the remaining 18 uL of the product were purified using GenElute Gel Extraction Kit (Sigma-Aldrich, USA), with a slightly modified protocol, to minimize inhibitors presence. After, purified genes concentrations were quantified using AccuGreen High Sensitivity dsDNA Quantitation Kit (Biotium) with a Qubit 2.0 Fluorometer (Thermo Fisher, USA). Thereafter, qPCR was performed on 5 dilutions of the purified product, with the calculation of the concentration of each being shown in Appendix A using the following mix: 10 uL Biotium Forget-Me-Not qPCR Master Mix, 1 uL of 5 uM primers, 1 uL of the template, and 7 uL of

molecular grade water for a total mix of 20 uL, ran on the CFX Connect Real-Time PCR Detection System (BioRad, USA). Melting curve analysis was performed by increasing temperatures from 65°C to 95°C at 0.5°C intervals in order to determine the amplicon specificity. Moreover, samples were run in triplicate in order to ensure results accuracy, and at least two blanks were used to ensure that no contaminations existed. Standard curves and amplification efficiency formulas can be found in Appendix B. The same thermocycling conditions as for the original standard PCR amplification were used for qPCR with the addition of a final elongation step. Thereafter, the results were normalized based on the sample type: influent (copies/mL), effluent (copies/mL), sludge biomass (copies/g.vss) or membrane (copies/cm^2). Moreover, standards for *rpoB* genes were created using the same above procedure, and concentrations were normalized to *rpoB* copies (Normalization formulas in appendix B).

Gene	Primers (5'-3')	Preincubation	Amplification	Cycles	Amplicon (bp)	Reference
sul1	F- CGCACCGGAAACATCGCTGCAC R- TGAAGTTCCGCCGCAAGGCTCG	95°C for 5 min	95°C for 30 s, 55 °C for 30 s, 72°C for 60 s	40	163	(Pei, Kim et al. 2006)
sul2	F- TCCGGTGGAGGCCGGTATCTGG R- CGGGAATGCCATCTGCCTTGAG	95°C for 5 min	95°C for 15 s, 56 °C for 30 s, 72°C for 40s	40	191	(Pei, Kim et al. 2006)
tetC	F-GCGGGATATCGTCCATTCCG R-GCGTAGAGGATCCACAGGACG	95°C for 5 min	95°C for 30 s, 55 °C for 30 s, 72°C for 60s	40	207	(Naas, Ergani et al. 2011)
tetQ	F- AGAATCTGCTGTTTGCCAGTG R-CGGAGTGTCAATGATATTGCA	95°C for 5 min	95°C for 30 s, 55 °C for 30 s, 72°C for 60 s	40	124	(Naas, Ergani et al. 2011)
ampC	F- CCTCTTGCTCCACATTTGCT R-ACAACGTTTGCTGTGTGACG	95°C for 5 min	95°C for 45 s, 58 °C for 60 s, 72°C for 60s	40	189	(Szczepanowski, Linke et al. 2009)
blaTEM	F-TTCCTGTTTTTGCTCACCCAG R-CTCAAGGATCTTACCGCTGTTG	95°C for 5 min	95°C for 45 s, 58 °C for 60 s, 72°C for 60s	40	445	(Bibbal, Dupouy et al. 2007)
intI1	F- CTGGATTTCGATCACGGCACG R-ACATGCGTGTAAATCATCGTCG	95°C for 5 min	95°C for 30 s, 60 °C for 60 s, 72°C for 60s	40	196	(Barlow, Pemberton et al. 2004)
tetW	F- GAGAGCCTGCTATATGCCAGC R- GGGCGTATCCACAATGTTAAC	95°C for 5 min	94°C for 45 s, 64 °C for 60 s, 72°C for 60s	40	167	(Masco, Van Hoorde et al. 2006)
tetA	F- GCTACATCCTGCTTGCCTTC R- CATAGATCGCCGTGAAGAGG	94°C for 5 min	94°C for 30 s, 55 °C for 30 s, 72°C for 60s	40	210	(Shi, Jia et al. 2013)
ereA	F- TCTCAGGGGTAACCAGATTGA R- TTATACGCAAGGTTTCCAACG	95°C for 10 min	95°C for 30 s, 58 °C for 30 s, 72°C for 60s	40	97	(Shen, Chu et al. 2019)

Mcr1	F- CGGTCAGTCCGTTTGTTC R- CTTGGTCGGTCTGTAGGG	95°C for 5 min	95°C for 45 s, 55 °C for 45 s, 72°C for 60s	40	309	(Tong, Liu et al. 2018)
rpoB	F- AACATCGGTTTGATCAAC R- CGTTGCATGTTGGTACCCAT	94°C for 5 min	94°C for 30 s, 50 °C for 90 s, 72°C for 90s	40	381	(Dahllöf, Baillie et al. 2000)

Table 1 Primers used for PCR and qPCR with the corresponding thermocycling conditions

Chapter Five

Results

5.1. Performance of the AnMBR during the municipal wastewater experiment

The performance of the AnMBR system was monitored throughout both phases of the experiment. The aim of the first phase was to increase the flux to achieve not only faster membrane fouling, but also to examine the effect of this increase on the ARG profiles in the permeate, as for the second phase, it aimed to highlight the effect of fouling by running fouled and unfouled membranes in parallel simultaneously. Throughout the experiment, the COD removal was 86.78 ± 3.62 % (Appendix B), and VFAs content was consistently below 10 mg/L and negligeable almost throughout the experiment (acetate, butyrate and propionate). As for the TSS and VSS, the first was 8.66 ± 0.48 g/L, and the latter was in the range of 7.2 ± 0.59 g/L, resulting in a VSS/TSS ratio of 0.83. As for the methane content, it remained relatively constant throughout the experiment at 90 ± 2.5 %, while the remaining gas consisted of carbon dioxide. Figure 3 illustrates the expected vs. produced methane in the reactor's headspace. Average actual methane produced was 540 ± 0.16 mL and was lower than the expected methane of 648 ± 0.25 mL, which was likely because of the increasing oversaturation ratios. The flux increase in the first phase did not have any effect on the above parameters nor did the membrane changes. The performance of the AnMBR system during the chicken slaughterhouse wastewater experiment is reported in detail in the master's thesis of Lama Ramadan entitled "Microbial and operational factors affecting energy recovery potential for anaerobic membrane bioreactors treating different wastewater types".

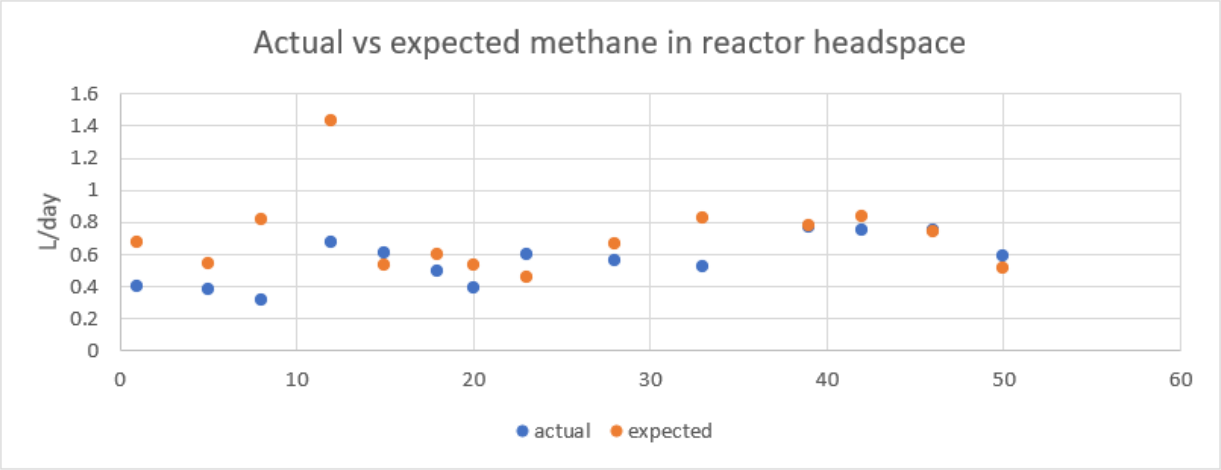

Figure 3 Expected vs actual produced methane in AnMBR headspace

5.2. Antibiotic resistance gene profiles in AnMBR treating poultry wastewater

As previously mentioned, AnMBRs are rich in different antibiotic resistance genes, as is chicken slaughterhouse wastewater (CSW) which is highly rich in ARGs due to the extensive use of antibiotics in this industry and the role they play as selective pressure. Moreover, due to the high SRT in AnMBRs, microbial communities in such systems are likely to adapt to the introduction of new wastewaters thus allowing for a compounded effect of HGT events (Noor, Rabiu et al. 2021). Figure 4 illustrate a sudden jump of ARG concentration in the effluent by nearly 2 log values after the introduction of CSW (Phase 1) to an AnMBR treating synthetic wastewater (startup phase). Afterward, this concentration was observed to decrease and stabilize in Phase 2 and Phase 3, which may be likely correlated to the adaptation of the AnMBR to the newly introduced CSW. The ARG composition of the CSW influent is illustrated in Figure 5 normalized per copy of *rpoB*, showing the rich composition of such wastewater in different type of intracellular ARGs that confers resistance to several classes of antibiotics. Thus, CSW introduction to the AnMBR resulted in high effluent ARG concentrations at first, which was likely the result of a spike in HGT events upon exposure of the AnMBR community to the new

influent microbiota. However, in the third phase, the system appeared to have recovered to achieve a more significant removal of such contaminants by 1-3 LRV.

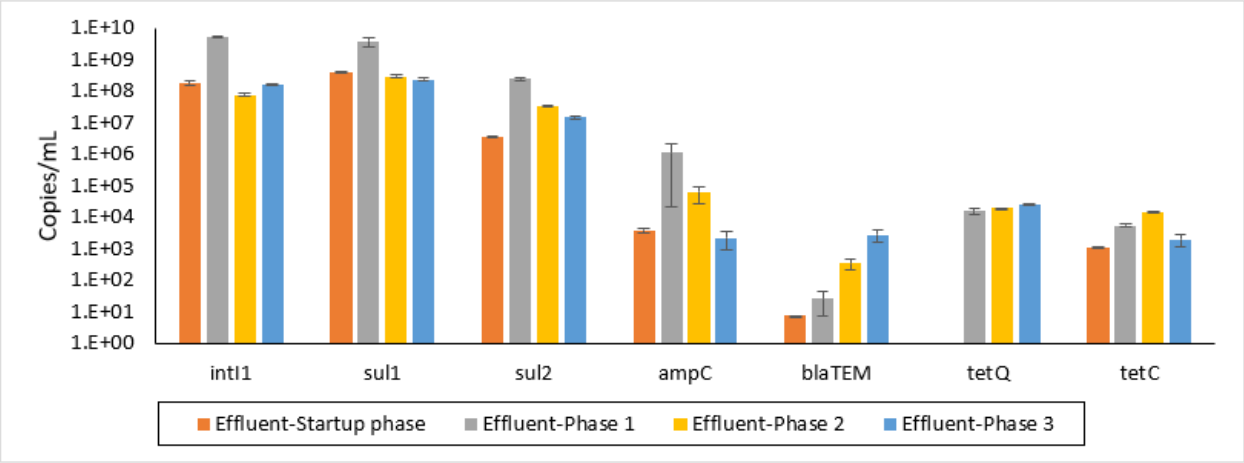

Figure 4 iARGs profiles in AnMBR treating poultry wastewater

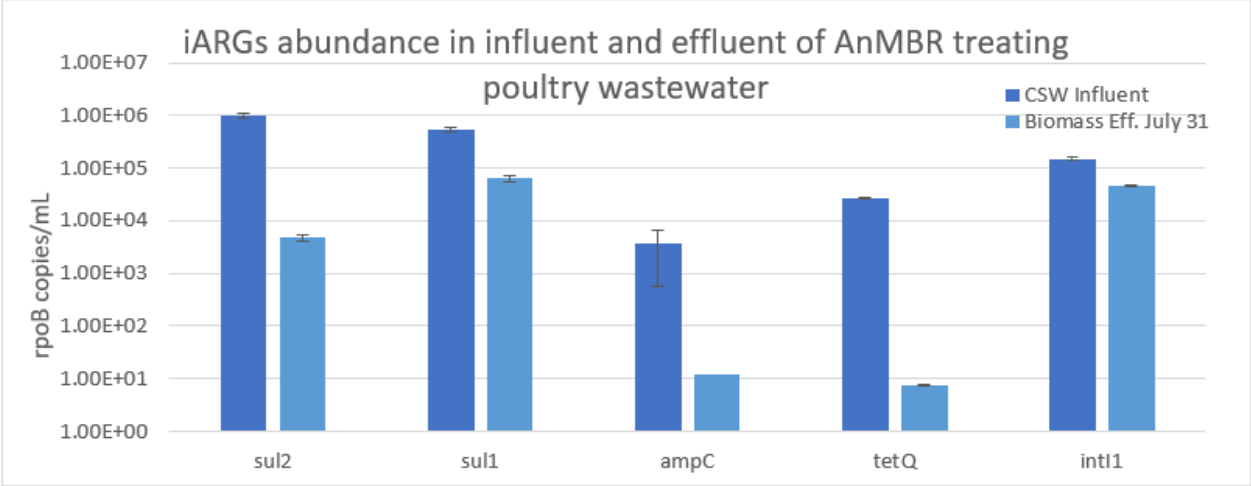

Figure 5 Chicken slaughterhouse composition in iARGs normalized by rpoB

In order to examine the effect of introduction of CSW to the suspended biomass (sludge) and on the membrane's ARG profiles, biomass and membrane samples were taken from the startup phase and Phase 3. Profiles shown in Figure 6 shows that with the exception of *blaTEM* and *intI1*, the introduction of CSW had a small impact (<1 LRV) on the suspended biomass composition of ARGs. As for the membranes samples illustrated in Figure 7, it was clear that

CSW introduction had a significant impact on *sul2*, *sul1*, *blaTEM*, *tetQ* and *intl1* genes profile. The above observations can be justified by the fact that microbial communities encompassing bacteria such as *Bacteroidetes*, *Sulfuricurvum*, and *Acinetobacter* were more diverse and richer in the membrane Phase 3 samples and less rich and diverse in the sludge. Thus, and noting that the previously mentioned bacteria are candidates that may enhance HGT of ARGs by being hosts (Da Silva and Domingues 2016, Groussin, Poyet et al. 2021), it may be concluded that membrane communities influenced ARG removal more than suspended biomass. This observation reiterates what was established by Zarei-Baygi et al. (2020), in which they deducted that membrane biofilms are better candidates for ARGs dissemination than suspended biomass.

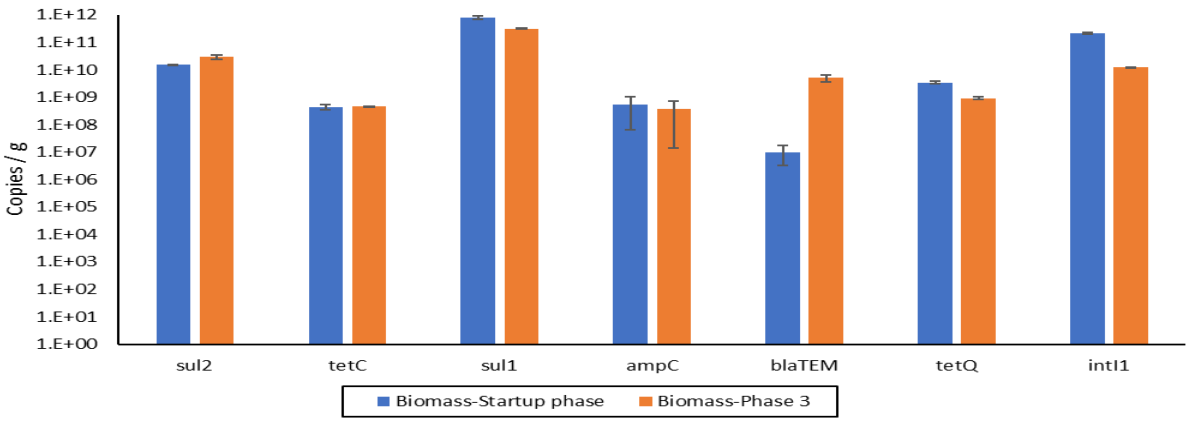

Figure 6 Effect of CSW introduction to the AnMBR on the suspended biomass (sludge)

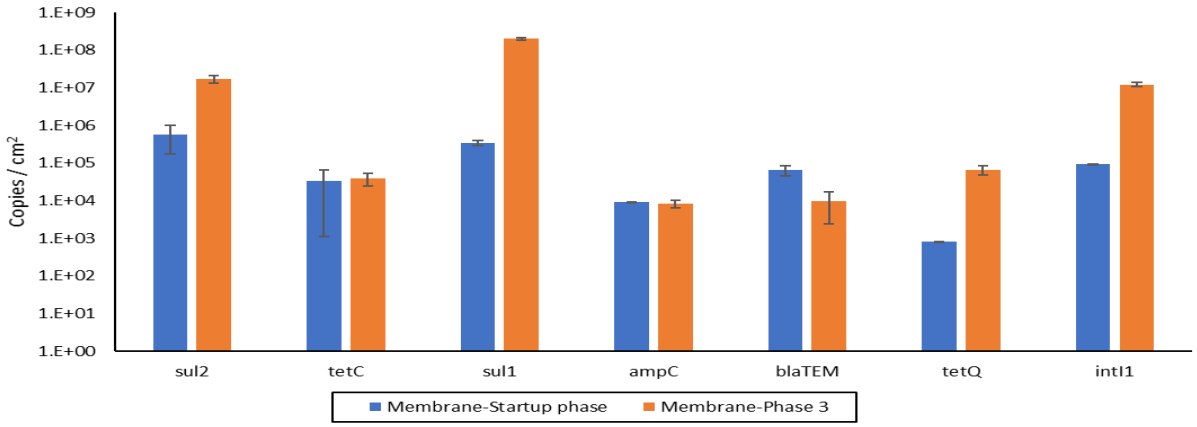

Figure 7 Effect of CSW introduction on the membrane biomass iARGs composition

5.2. Antibiotic resistance gene profiles in AnMBR after introduction of municipal wastewater

A transition from synthetic feed to real wastewater influent was performed on the system after being fed only synthetic feed for over 6 months in a startup phase in order to analyze the effect of introducing real municipal wastewater on ARGs profiles in the AnMBR's effluent. ARG concentrations were determined using qPCR on influent and effluent samples ran in triplicate after the municipal wastewater influent was introduced to the AnMBR. Characteristics of the wastewater are summarized in Table 2 with a COD of 578 ± 68 mg/L. Figure 8 shows the *rpoB* gene copies present in the influent and effluent and indicates an increase in bacterial abundance in the effluent after municipal wastewater introduction. Moreover, Figure 9 shows that not only was the municipal wastewater rich in bacterial abundance, but also in ARGs that confers resistance to a wide variety of antibiotic classes, such as sulfonamide, tetracycline, ampicillin and beta-lactams. Moreover, class 1 integron gene, *intl1*, was abundant, indicating the presence of mobile genetic elements responsible for HGT and proliferation of ARGs in the AnMBR effluent. It was observed over time that the concentrations of almost all iARGs, except for *ampC*

and *blaTEM* increased with the introduction of municipal wastewater (Figure 9) i.e., between day 1 and day 42 of the experiment, with day 1 corresponding to the day prior to the introduction of municipal wastewater. Previous studies observed similar iARGs as were detected in this work, with the same concentration ranges of 10^3 -10^8 copies /mL in municipal wastewaters (Kappell, Kimbell et al. 2018, Le, Ng et al. 2018, Zhang, Yang et al. 2018). As for the ability of AnMBR to remove iARGs from municipal wastewater, it was observed that LRV ranged from 1 to 4 and removals, even if minimal, happened for all iARGs, with *sul1*, *intl1*, and *sul2* having the least removal rates. Noting that the above genes are all intracellular genes, and thus hosted or already present in bacteria, events of HGT may have been responsible for the observed increases in several of the iARG concentrations over prolonged exposure to the municipal wastewater influent (Figure 9). For instance, it can be observed that *intl1*, *sul1*, and *sul2* gene copies increased in parallel overtime, and almost became of the same magnitude as the influent, suggesting that sulfonamide resistance genes propagated thru horizontal gene transfer into the effluent. Moreover, their increase in parallel may be correlated to the presence of *sul1* and *sul2* genes on gene cassettes associated with class 1 integrons, as was observed in previous studies (Frank, Gautier et al. 2007, Wu and Kim 2020).

Table 2 Influent municipal wastewater composition

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

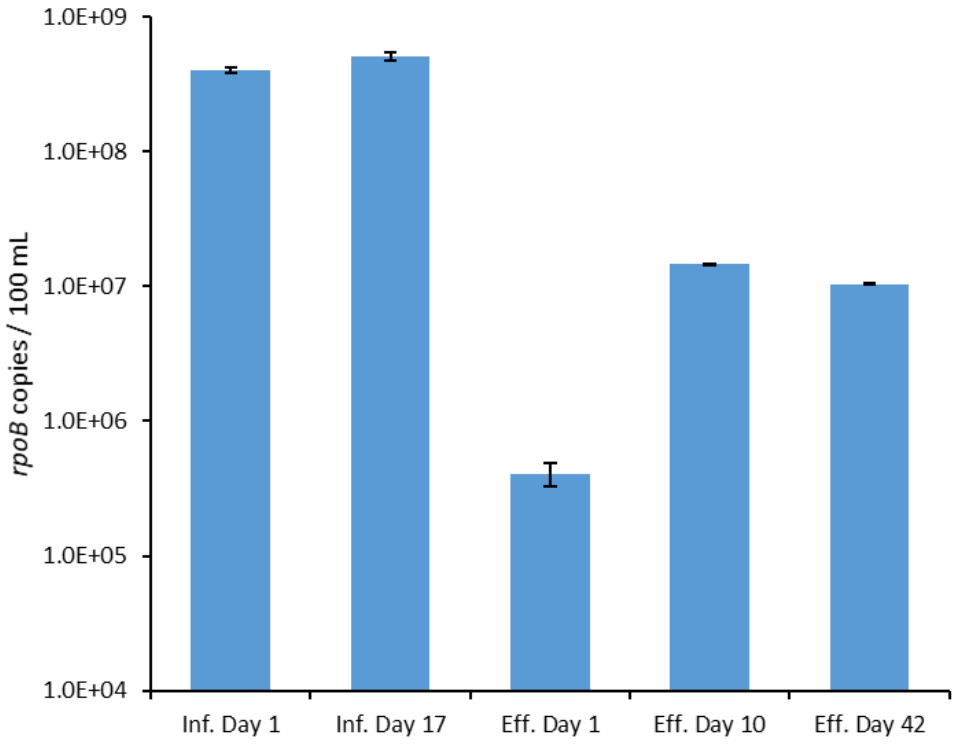

Figure 8 rpoB copies present in municipal wastewater and AnMBR effluent after introduction of MWW

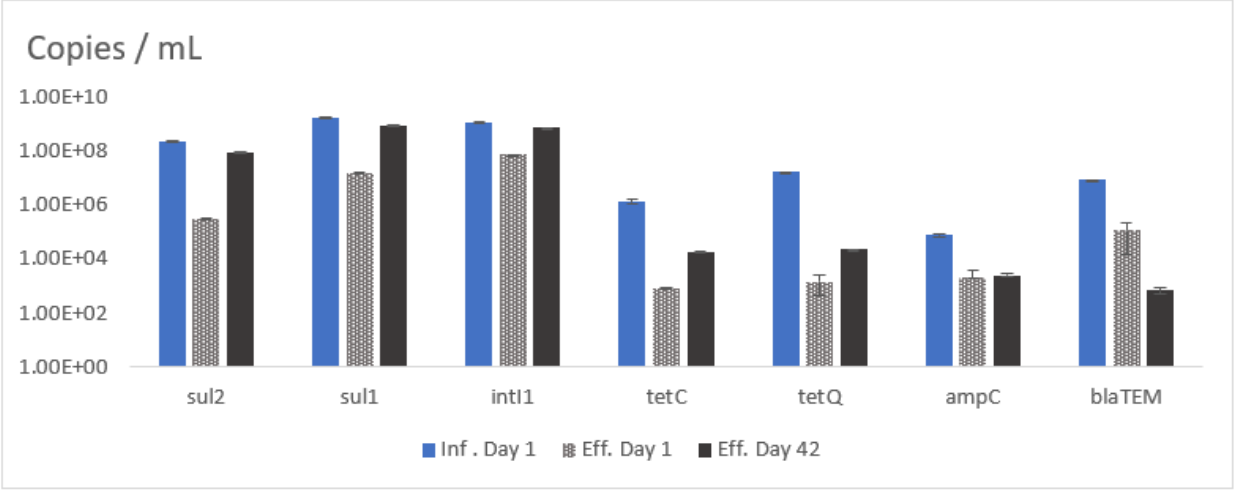

Figure 9 iARGs profile in AnMBR effluent treating MWW influent

5.3. Extracellular and intracellular antibiotic resistance gene profiles in the AnMBR treating municipal wastewater

Samples taken from municipal wastewater treatment plants over two major locations in Lebanon were found to be rich in not only intracellular, but also extracellular ARGs as shown in Figure 10. Moreover, all ARGs were found in the extracellular form more abundantly than in the intracellular form, which may increase the frequency of HGT events making ARG proliferation in the AnMBR effluent a more concerning threat. This was further accentuated by the presence of mobile genetic elements such as the class 1 integron integrase gene (*intl1*), which was found to be present in high concentrations (Sui, Chen et al. 2019). Sulfonamide, beta-lactam, tetracycline, colistin, ampicillin, and erythromycin resistance genes were found in large concentrations of 10^2 to 10^8 copies per mL in the intracellular form, and 10^4 to 10^{12} in the extracellular form. In addition, it was observed that the AnMBR achieved 1 to 2 LRV for ARGs in both forms, however, it can be noticed that for some genes such as *tetW* and *intl1*, eARG removal was achieved while iARG wasn't. This can possibly indicate that for instance extracellular *tetW* genes, underwent HGT and thus were present as intracellular *tetW* in the effluent (Figure 11). Moreover, as shown in Figure 10, *mcr1* and *tetQ* genes were not detected in the effluent in the extracellular form, although they are present in the influent, what can indicate that those genes underwent HGT in the AnMBR suspended biomass or membranes, considering that both genes have high affinity of plasmid mediated HGT, and can undergo extensive natural gene transmission (Nikolich, Hong et al. 1994, Yamamoto, Higashi et al. 2022). Plus, it can be observed that in the effluent, eARGs concentrations were higher by 2-7 log values than iARGs, which was observed as well by Sui et. Al (2019), after MBR process, what implies slower degradation rates and longer persistence of plasmid-borne ARGs.

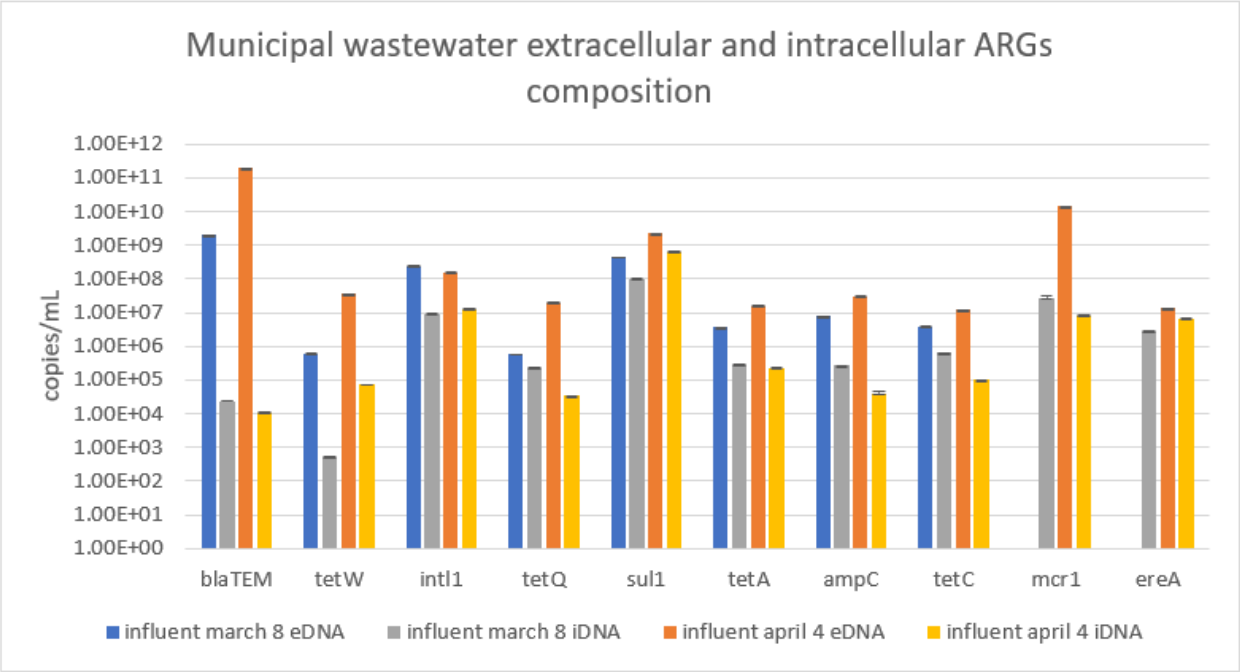

Figure 10 eARGs and iARGs profile in municipal wastewater influents

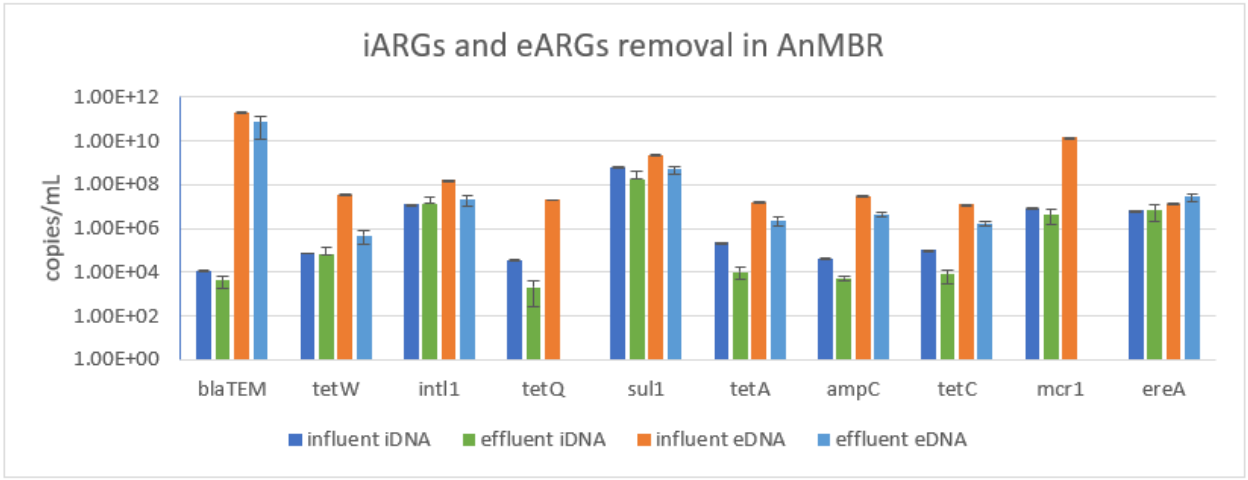

Figure 11 iARGs and eARGs profiles in AnMBR effluents treating municipal wastewater

5.4. Effect of changing AnMBR operating conditions on iARG and eARG dynamics

Variables in the AnMBR operating conditions were tested for their effect on intracellular and extracellular profiles and dynamics in AnMBR permeate, as well as their removal efficiency. Those variables included flux variations, use of different membrane pore sizes and membrane fouling. As observed by researchers previously, such parameters can affect ARG dynamics in MBR systems (Harb, Wei et al. 2016, Li, Yuan et al. 2019, Sui, Chen et al. 2019).

5.4.1. Effect of flux variation on eARGs and iARGs profiles in the effluent

The AnMBR's operational transmembrane flux was manipulated by changing the effluent pumping rate throughout the first phase of the experiment, starting by 0.8 mL/min up to 1.4 mL/min in 0.2 mL/min increments. As the critical flow of the AnMBR was previously observed to be 1.2 mL/min, the results were divided to pre flux increase phase, which corresponds to the 0.8 mL/min and 1 mL/min flow, and post flux increase corresponding to 1.2 mL/min and 1.4 mL/min flow rates. Figure 12 shows the flow rate increases throughout the experiment. This can be confirmed by Figure 13, as it can be seen that for both membranes, a considerable increase in transmembrane pressure (TMP) was noticed at a flow rate of 1.2 mL/min, thus transmembrane pressure started to play a role at this flow rate.

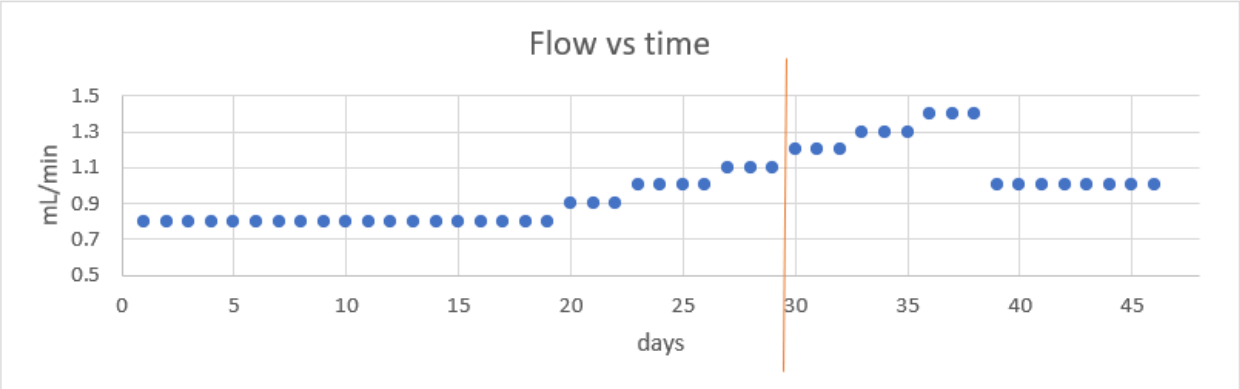

Figure 12 Pumping Flow rate throughout the experiment

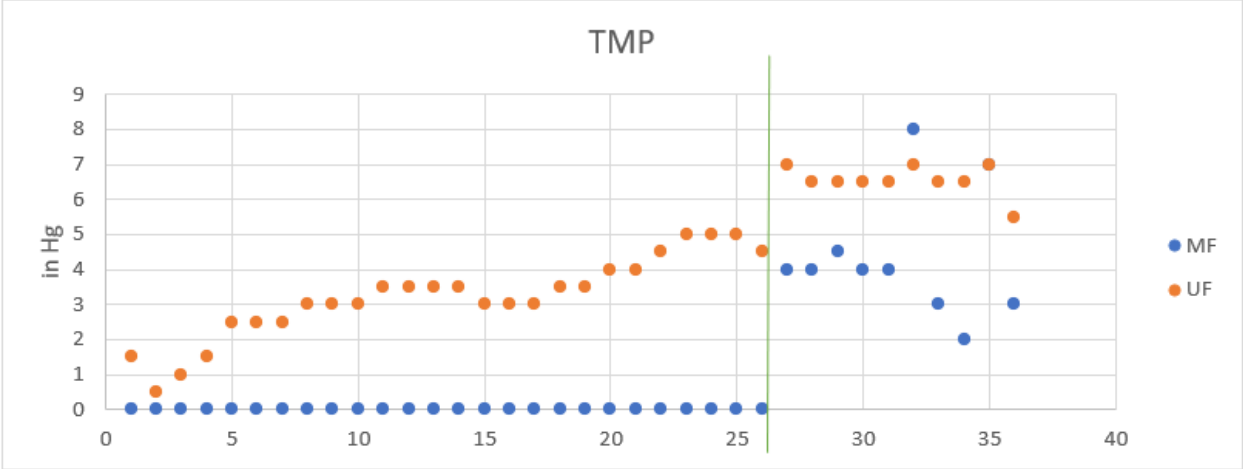

Figure 13 Transmembrane pressure for UF and MF membranes

It can be observed from Figures 14, 15 and 16 that after flux increase for both ultrafiltration and microfiltration membranes, the trend that iARGs followed was to increase for all ARGs with the exception of *tetA*. This observation may be because as the flow rate increased, the transmembrane membrane pressure (TMP) increased, thus the membranes permitted more passage of the cells, which was also observed by Zarei-Baygi et al. (2020), for which in their experiment the membrane with the highest TMP resulted in the highest concentration of iARGs in the permeate.

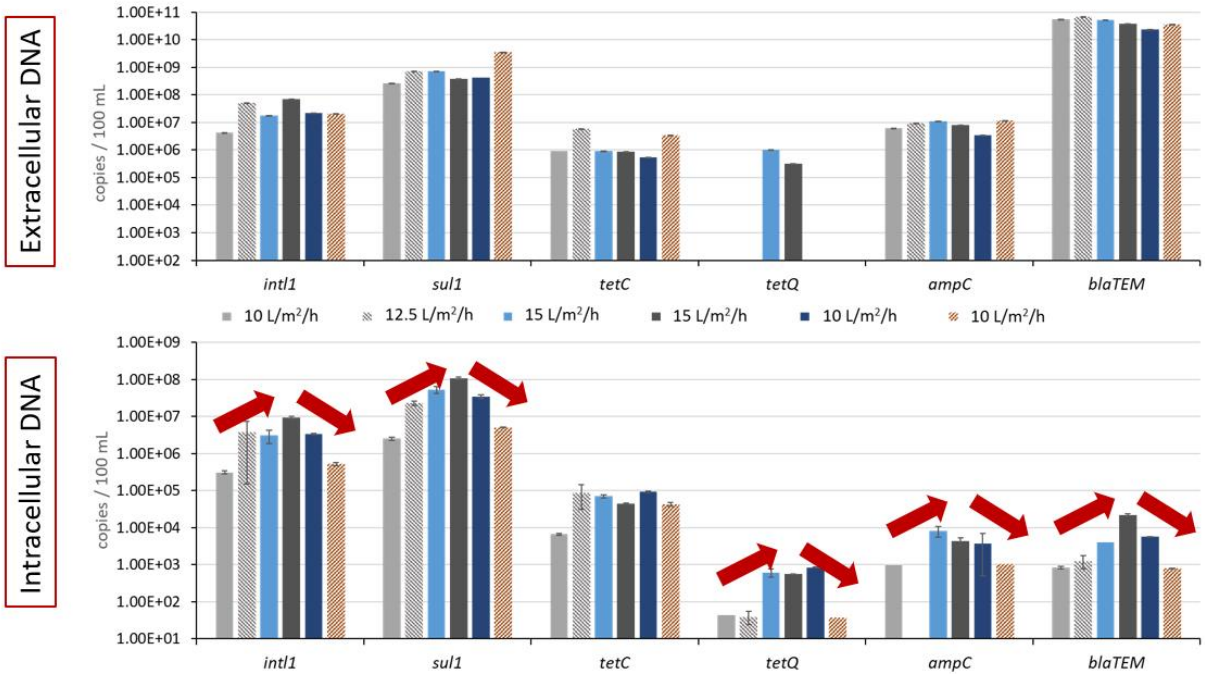

Figure 14 Extracellular and intracellular ARGs trend with flux increase in AnMBR treating real MWW

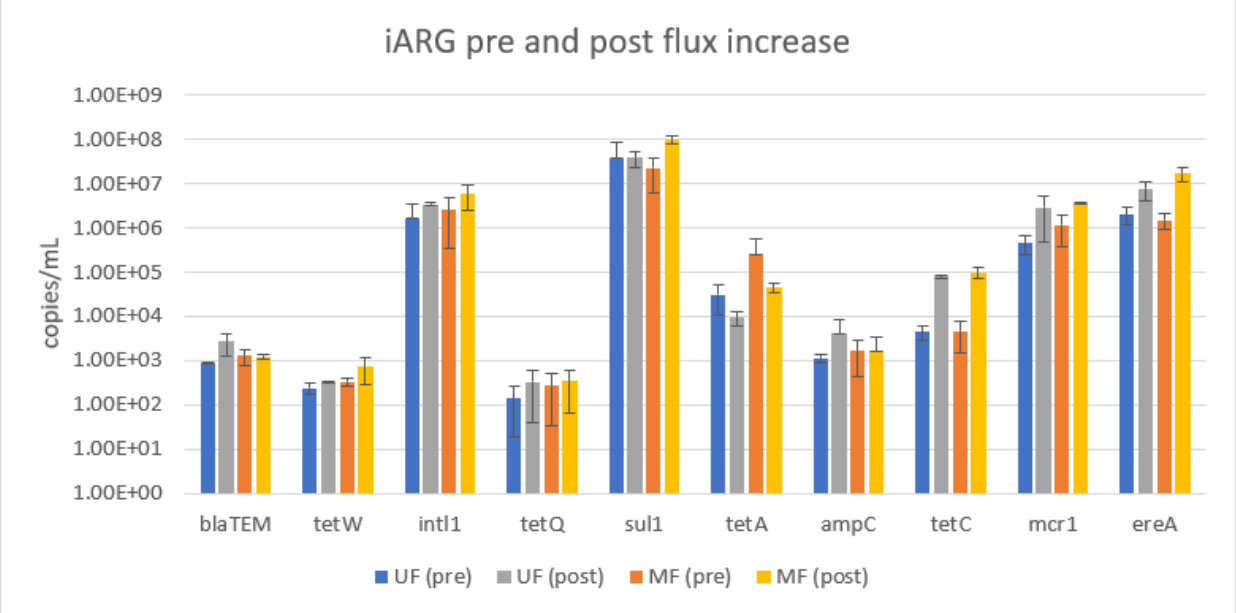

Figure 15 intracellular ARGs in UF and MF membrane permeate pre and post flux increase

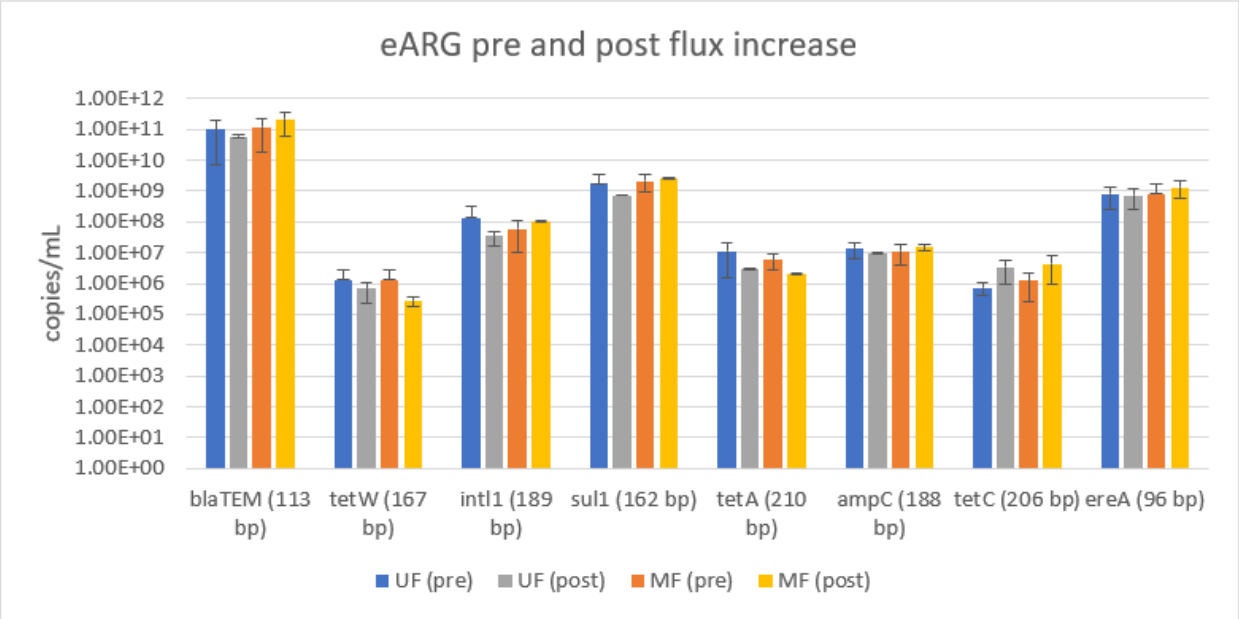

Figure 16 extracellular ARGs in UF and MF membrane permeate pre and post flux increase

As for eARGs, no clear trend was observed for the MF membrane, however, for the UF membrane, a slight decrease can be noticed for all eARGs post flux increase with the exception of *tetC*. This may be related to the large size of *tetC* (200 bp), leading to pressure having more of an effect on it and squeezing the free floating genes into membrane pores, which was observed with iARGs as well. Moreover, TMP had a more intense effect on the UF membrane than on the MF membrane regarding eARG trends, which can be related to the smaller pores of the UF membrane retaining more extracellular genes than MF membrane (in which permeate contained almost same concentrations of eARGs pre and post increase). Thus, the large size of MF membrane pores and the small size of the free-floating genes made the effect of flux increase and TMP negligeable. For instance, it can be seen that for the smallest genes, i.e., *ereA*, *blaTEM*, and *sul1*, their concentrations in extracellular form increased post flux increase in the MF membrane permeate, which can, as well, be attributed to the membrane large pore sizes.

Thus, TMP played an important role in the dynamics of ARGs, and their removal. For the UF membrane, iARGs concentrations increased while eARGs concentrations decreased, thus mitigating the removal effects. Resultantly, for the UF membrane, LRVs were not affected by the TMP and flux, which was also observed by Zarei-Baygi (2020). However, for the MF membrane with larger pore sizes, both iARGs and eARGs concentrations in the permeate increased, resulting in overall lower LRVs for the total ARGs. Moreover, flux increase effect on ARGs dynamics may not only be limited to the physical pressure pushing genes and pathogens through membrane pores but also the membrane-gene contact time, which can affect the ability of the membrane to adsorb those genes or even for HGT to happen based on the HGT frequency in such situations.

5.4.2. Effect of fouling on eARGs and iARGs profiles in the effluent

Another factor that was found to alter ARGs dynamics is membrane level of fouling, thus flux was increased to let membranes foul faster. Fouling was achieved after the critical flux of 1.2 mL/min, as TMP started to rise gradually.

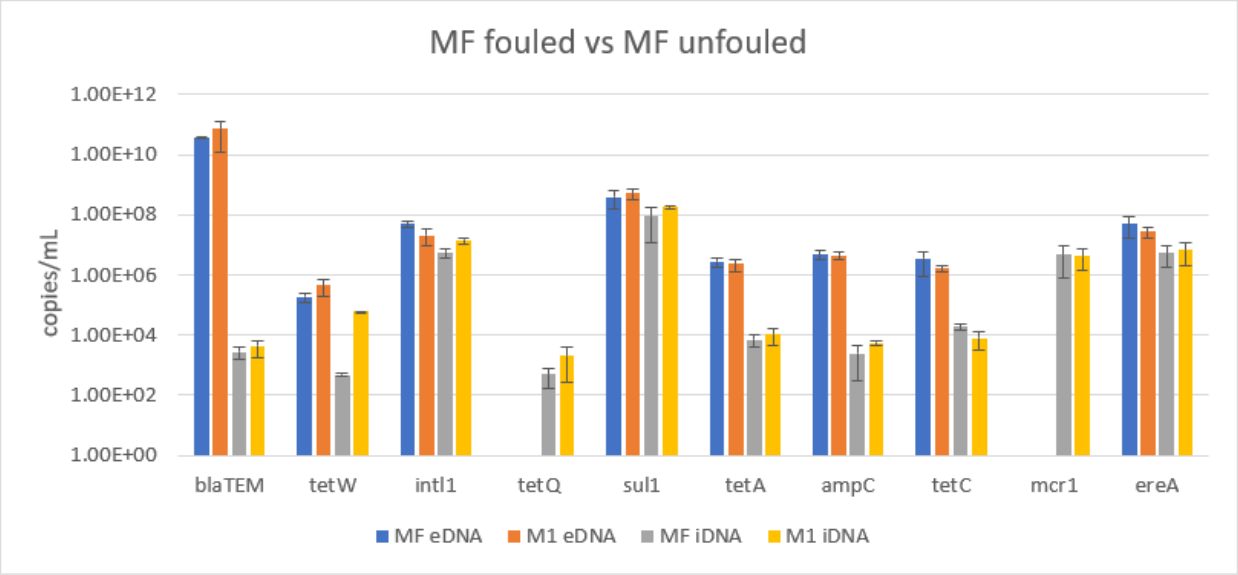

Figure 17 iARGs and eARGs presence in fouled and unfouled MF membrane permeate

First, the effect of fouling on MF membrane was studied (Figure 17), where MF represent the fouled microfiltration membrane whereas M1 represent the unfouled one. It can be seen that for eARGs, no trend was observed on the effect of fouling regarding eARGs removal, which can be related to the previous cause, where MF membrane pores are still large enough (even with fouling) to let eARGs through and the minimal effect of biofilms in HGT events in this case. For iARGs (with the exception of *tetC*), the fouled MF membrane achieved only slightly better removal than the unfouled membrane, which may be attributed to physical filtration removal of cells as the flux was below critical in this phase (1 mL/min), thus, no iARGs were squeezed through the membrane.

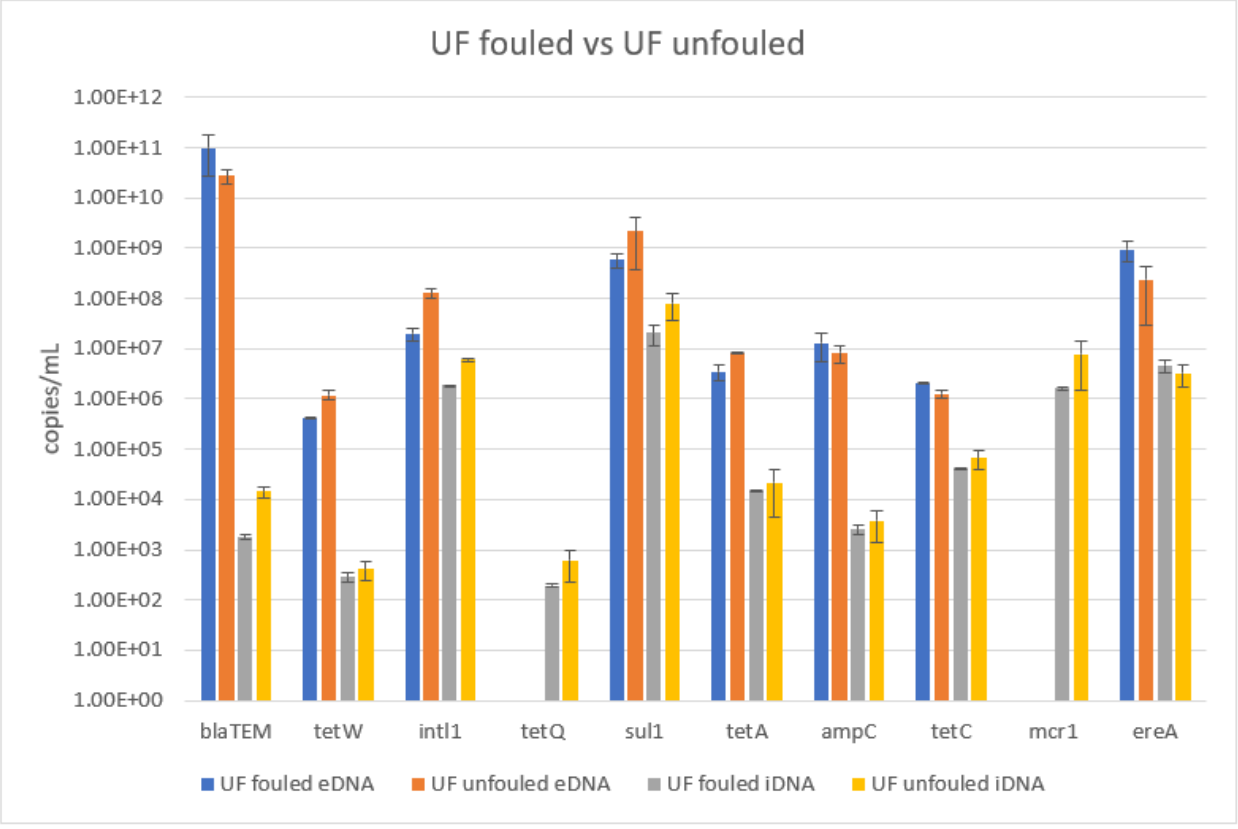

Figure 18 iARGs and eARGs presence in fouled and unfouled UF membrane permeate

Second, for the ultrafiltration membrane, extracellular gene concentrations in the permeate of the unfouled membrane were 0.5 to 2 log values higher than the fouled one for several genes including *tetW, intl1, sul1*, and *tetA*. On the other hand, *blaTEM*, *ereA,* and *tetC* showed an increase in their extracellular form after fouling, however, those genes are the smallest and therefore were likely to be least impacted by membrane fouling. Moreover, *blaTEM* and *ereA* are usually found in extracellular form more abundantly than intracellular form, which also may explain that fouling did not lower their concentrations, as HGT events to membrane biofilms maybe negligeable for those genes (Sui, Chen et al. 2019, O'Malley, McDonald et al. 2022).

As for ARGs in the intracellular form, a consistent decrease in the concentrations was achieved after fouling, ranging as well from 1 to 2 LRV. Moreover, fouling effect had a more significant effect on iARGs removal from the UF membrane compared to the MF membrane, which may be due to the smaller size of the UF pores, thus the fouled UF membrane achieved better ARG removal than the fouled MF membrane for iARGs in particular and thus the overall removal as total ARGs.

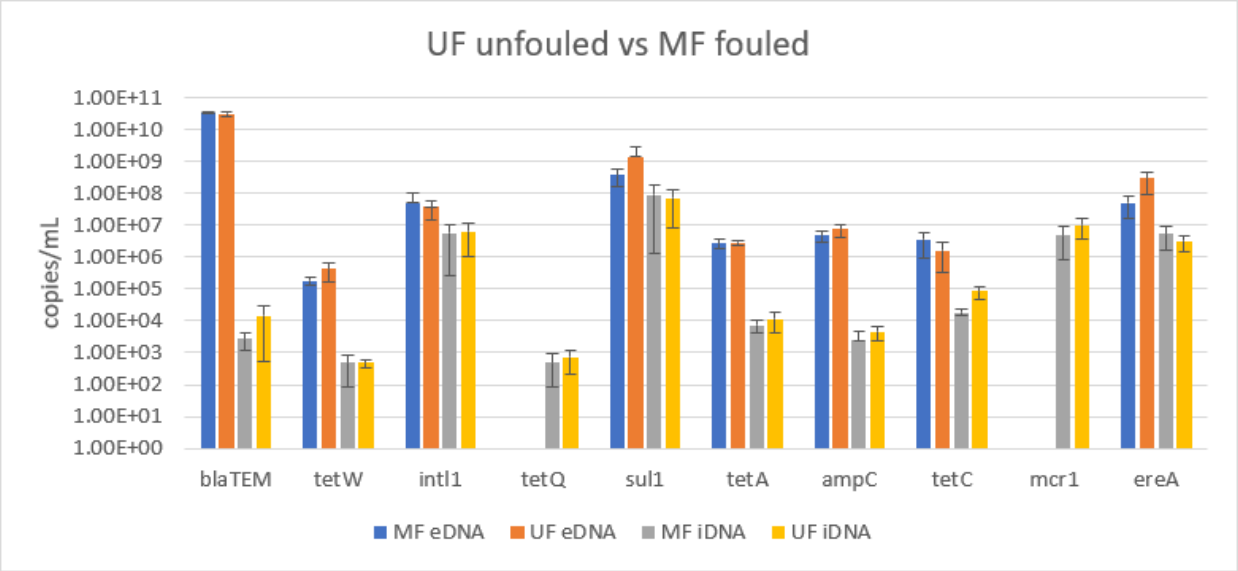

Figure 19 iARGs and eARGs presence in perameate of fouled MF membrane vs unfouled UF membrane

Third, comparing ARG removal efficiency of a fouled MF membrane to an unfouled UF membrane (Figure 19), it was observed that for both extracellular and intracellular ARGs, a fouled MF membrane achieved similar or better LRV for most genes. Similar results were observed by Cheng and Hong (2017), as a positive correlation between membrane fouling and ARG LRVs was seen.

Finally, it was observed that that a fouled MF membrane operating at a high TMP (with a flux above the critical one) had higher extracellular ARGs in its permeate than a fouled MF membrane at low TMP for all ARGs. For intracellular ARGs for all ARGs except *blaTEM*, *ampC*, *tetQ* and *mcr1*, the fouled MF membrane at high TMP also achieved lower LRV than a fouled MF membrane at low TMP (Figure 20). Thus, the TMP appeared to be of equal importance as membrane biofilm presence in ARG removal efficiency. Those results are consistent with Cheng and Hong (2017) results, which indicated that as membranes became fouled, a higher TMP resulted in a lower LRV of ARBs.

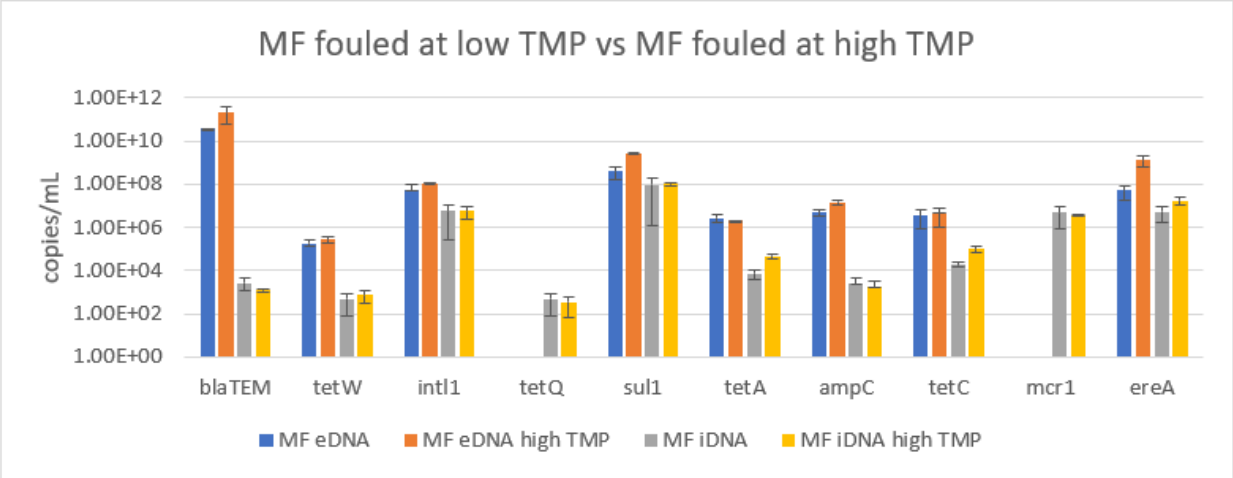

Figure 20 TMP and fouling effects on iARGs and eARGs profiles in MF permeates

Chapter Six

Discussion

The main goal of this study was not only to examine the ability of AnMBRs treating real municipal wastewater in the removal of extracellular and intracellular ARGs, but also to understand the effect of flux, membrane sizes and fouling levels on the composition of such contaminants in the permeate. Moreover, this study contributed to the understanding of the effects of the introduction of MWW and CSW on AnMBRs in terms of ARG profiles. First, among the parameters that were tested was flux variation and TMP effect on the permeate composition of iARGs and eARGs for both an MF and UF membranes. For that matter, the effect of TMP and flux increase was not only different on eARGs and iARGs, but also was found to differ between each membrane with different pore sizes. For instance, it was confirmed that eARGs and iARGs were inversely affected by flux increase for the UF membrane, as flux increase resulted in the increase of iARGs in the UF permeate, but the decrease of certain eARGs. For the MF membrane, iARGs concentrations increased in the permeate while eARGs did not seem to follow a trend. As this variation could be correlated to the membrane pore sizes (and possibly HGT events), more studies are required to examine the membranes composition effects on eARGs and iARGs, OTUs, and microbial community to get more insights about possible HGT events. Thus, TMP play a key role in the performance of AnMBR in treating ARGs and is affected by the form in which those genes exist, in addition to the membrane pore sizes themselves.

Second, another parameter affecting ARG profiles in AnMBR's permeate is the presence of biofilms on the membranes. For instance, it was found that membrane size is as well an important parameter even with membrane fouling. However, in this experiment a similar level of fouling was achieved for the UF and MF membranes, thus further studies with different extent of fouling should be accomplished. It was found that a fouled UF membrane achieved better performance in removal of both eARGs and iARGs than an unfouled UF membrane, however, a fouled MF membrane only achieved a similar to slightly higher removal of iARGs than an unfouled MF membrane, with eARGs removal being unaffected. Moreover, a comparison between a fouled MF membrane and a non-fouled UF showed that a fouled MF membrane can achieved similar and occasionally better removal of both eARGs and iARGs than an unfouled UF membrane. This may be attributed to both the effect of HGT in the membrane biofilms (which needs to be further investigated) and the fact that fouling makes the pores of the membrane physically smaller, thus it would be interesting to further investigate the best fouling extent to achieve such removal performance, while minimizing TMP and energy requirements. Moreover, it is worth noting that not only a fouled MF membrane can achieve same or better removal than a UF membrane, but also the fouled MF membrane had larger effluent methane production throughout the experiment compared to the UF (23.8 mL/L of effluent vs 17.0 mL/L of effluent), thus having an added need for applying downstream methane recovery systems.

Third, it was found that TMP and membrane fouling have a similar weight in terms of AnMBR performance in the removal of ARGs, especially with an MF membrane. Therefore it is critical to establish studies that examine the extent of fouling to achieve an optimal balance between TMP and level of biofilm development. For instance, it was observed that a fouled MF membrane at low TMP achieved greater ARG LRVs than a fouled MF membrane operated at

high TMP. Furthermore, in this study it was observed that TMP and fouling didn't quite have an important role on the UF membrane, however, it is important to consider the amount of energy needed to operate a fouled UF membrane at high TMP. Thus, it was found that an MF membrane operating at low TMP can be an optimal option while considering ARGs removal performance and energy recovery.

Fourth, it was found that the transition from synthetic feed to MWW and CSW caused a sudden increase in the ARGs present in the system. This increase was found to be more critical in the membranes rather than the suspended biomass. This difference between the two components of the system might be related to several factors, that include the sludge concentration in ARGs, thus the membranes having more room to absorb those contaminants, or simply because the membranes biofilms have more HGT capabilities as they encompass OTUs that are different from the sludge. Finally, it was observed that ARGs were successfully removed from the system after some time from which CSW and MWW influent were introduced. This removal after some time may be attributed to the possibility that the microbial communities formed in the system and the membranes adapted to the influent being introduced.

The present work aimed to examine the ability of AnMBR systems to treat various types of wastewaters for ARGs. It was apparent that such systems are generally effective at lowering abundances of various types of wastewaters rich in ARGs, and that this efficiency is impacted by the operating conditions. However, a limitation of this study is the lack of information about the biofilms developed on the membranes, for instance, the thickness of those layers, microbial communities and ARGs present on them should have been examined for a further understanding of the HGT and physical filtration effects on ARGs removal. Moreover, testing for different level of fouling, membrane sizes and TMP combinations for the removal of ARGs from municipal

wastewater while also performing a net energy balance on those combinations can help in finding the best combination for ARG LRVs and highest energy recovery. Moreover, another limitation of this study was the low recovery rate of extracellular ARGs, by the lack of the proper equipment needed for this application. For instance, 0.45 um membrane filters were used rather than the 0.22 um ones, in addition to other missing equipment that resulted in precipitated pellet losses. This loss was mitigated by normalizing the values to the spiked plasmid, however, the recovery efficiency was still low.

Chapter Seven

Conclusions

The proliferation of ARGs around the world has been identified as a serious threat, which limits the ability of wastewater reuse for irrigation and other purposes and, in turn, amplifies water scarcity issues. AnMBRs have been recognized as a promising technology with potential for ARG removal from various types of wastewaters while having high energy recovery potential. Thus, this experiment focused on the ability of AnMBRs to remove eARGs and iARGs from municipal wastewater, which is one of the largest contributors to wastewater discharges worldwide (volumetrically) and among the richest in such contaminants. The two main phases of the experiment aimed to examine the effect of manipulating system flux, use of different membrane sizes, TMP, and fouling effect on the removal of iARGs and eARGs. The present work suggested that the previously mentioned parameters can have different effects on ARG dynamics for each membrane. The study proved that a fouled MF membrane can achieve similar LRVs for both eARGs and iARGs as an unfouled UF membrane while potentially requiring less energy for operation. The study is the first (to the authors knowledge) not only to examine the LRVs of iARGs and eARGs by AnMBRs treating real municipal wastewater using multiple membrane pore sizes, but to assess the changes in critical operating conditions on the removal efficiency as well.

7.1. Future Work

Targeted research for understanding the ability of AnMBRs to remove emerging contaminants from different wastewaters are still an understudied topic in all aspects. As those systems have been found to be effective in achieving certain LRV of ARGs, the establishment of risk analysis models such as QMRA is of great importance so as to have a tool for quantifying if the removal efficiency is sufficient for downstream applications of effluents (such as crops irrigation). Moreover, post treatment strategies that focus extensively on ARGs and other contaminants removal must be established as, even with no risk assessment models, it is clear that AnMBR effluents are rich in ARGs for which dominant proliferation, HGT mechanisms and mechanisms of action are still unknown in most environments.

References

Amarasiri, M., et al. (2020). "Understanding human health risks caused by antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARG) in water environments: Current knowledge and questions to be answered." Critical Reviews in Environmental Science and Technology **50**(19): 2016-2059.

Augsburger, N., et al. (2021). "The use of UV/H2O2 to facilitate removal of emerging contaminants in anaerobic membrane bioreactor effluents." Environmental Research **198**: 110479.

Aziz, A., et al. (2022). "Anaerobic digestion in the elimination of antibiotics and antibiotic-resistant genes from the environment–A comprehensive review." Journal of environmental chemical engineering **10**(1): 106423.

Baek, S. H. and K. R. Pagilla (2006). "Aerobic and anaerobic membrane bioreactors for municipal wastewater treatment." Water environment research **78**(2): 133-140.

Barlow, R. S., et al. (2004). "Isolation and characterization of integron-containing bacteria without antibiotic selection." Antimicrobial Agents and Chemotherapy **48**(3): 838-842.

Berendonk, T. U., et al. (2015). "Tackling antibiotic resistance: the environmental framework." Nature Reviews Microbiology **13**(5): 310-317.

Bibbal, D., et al. (2007). "Impact of three ampicillin dosage regimens on selection of ampicillin resistance in Enterobacteriaceae and excretion of bla TEM genes in swine feces." Applied and environmental microbiology **73**(15): 4785-4790.

BouNehme Sawaya, C. and M. Harb (2021). "Considering the prospect of utilizing anaerobic membrane biofouling layers advantageously for the removal of emerging contaminants." Frontiers in Chemical Engineering: 2.

Breazeal, M. V. R., et al. (2013). "Effect of wastewater colloids on membrane removal of antibiotic resistance genes." Water research **47**(1): 130-140.

Cheng, D., et al. (2018). "Anaerobic membrane bioreactors for antibiotic wastewater treatment: performance and membrane fouling issues." Bioresource technology **267**: 714-724.

Cheng, H., et al. (2019). "Identification and characterization of core sludge and biofilm microbiota in anaerobic membrane bioreactors." Environment international **133**: 105165.

Cheng, H. and P.-Y. Hong (2017). "Removal of antibiotic-resistant bacteria and antibiotic resistance genes affected by varying degrees of fouling on anaerobic microfiltration membranes." Environmental science & technology **51**(21): 12200-12209.

Czekalski, N., et al. (2014). "Wastewater as a point source of antibiotic-resistance genes in the sediment of a freshwater lake." The ISME journal **8**(7): 1381-1390.

Da Silva, G. J. and S. Domingues (2016). "Insights on the horizontal gene transfer of carbapenemase determinants in the opportunistic pathogen Acinetobacter baumannii." Microorganisms **4**(3): 29.

Dagnew, M. and W. Parker (2021). "Impact of AnMBR operating conditions on anaerobic digestion of waste activated sludge." Water environment research **93**(5): 703-713.

Dahllöf, I., et al. (2000). "rpoB-based microbial community analysis avoids limitations inherent in 16S rRNA gene intraspecies heterogeneity." Applied and environmental microbiology **66**(8): 3376-3380.

Dever, L. A. and T. S. Dermody (1991). "Mechanisms of bacterial resistance to antibiotics." Archives of internal medicine **151**(5): 886-895.

Dong, P., et al. (2019). "Assessment of extracellular antibiotic resistance genes (eARGs) in typical environmental samples and the transforming ability of eARG." Environment international **125**: 90-96.

Fatta-Kassinos, D., et al. (2011). "The risks associated with wastewater reuse and xenobiotics in the agroecological environment." Science of the Total Environment **409**(19): 3555-3563.

Frank, T., et al. (2007). "Characterization of sulphonamide resistance genes and class 1 integron gene cassettes in Enterobacteriaceae, Central African Republic (CAR)." Journal of antimicrobial chemotherapy **59**(4): 742-745.

Gonzalez-Gil, L., et al. (2017). "Cometabolic enzymatic transformation of organic micropollutants under methanogenic conditions." Environmental science & technology **51**(5): 2963-2971.

Gonzalez-Gil, L., et al. (2018). "Role of methanogenesis on the biotransformation of organic micropollutants during anaerobic digestion." Science of the Total Environment **622**: 459-466.

Groussin, M., et al. (2021). "Elevated rates of horizontal gene transfer in the industrialized human microbiome." Cell **184**(8): 2053-2067. e2018.

Guo, W., et al. (2020). Anaerobic membrane bioreactors for antibiotic wastewater treatment. Current Developments in Biotechnology and Bioengineering, Elsevier**:** 219-239.

Hamilton, A. J., et al. (2006). "Quantitative microbial risk assessment models for consumption of raw vegetables irrigated with reclaimed water." Applied and environmental microbiology **72**(5): 3284-3290.

Harb, M. and P.-Y. Hong (2017). "Anaerobic Membrane Bioreactor Effluent Reuse: A Review of Microbial Safety Concerns." Fermentation **3**(3): 39.

Harb, M. and P.-Y. Hong (2017). "Molecular-based detection of potentially pathogenic bacteria in membrane bioreactor (MBR) systems treating municipal wastewater: a case study." Environmental Science and Pollution Research **24**(6): 5370-5380.

Harb, M., et al. (2016). "Organic micropollutants in aerobic and anaerobic membrane bioreactors: Changes in microbial communities and gene expression." Bioresource technology **218**: 882-891.

Inaba, T., et al. (2020). "Microbial community in an anaerobic membrane bioreactor and its performance in treating organic solid waste under controlled and deteriorated conditions." Journal of Environmental Management **269**: 110786.

Kanafin, Y. N., et al. (2021). "Anaerobic membrane bioreactors for municipal wastewater treatment: a literature review." Membranes **11**(12): 967.

Kappell, A. D., et al. (2018). "Removal of antibiotic resistance genes in an anaerobic membrane bioreactor treating primary clarifier effluent at 20 C." Environmental Science: Water Research & Technology **4**(11): 1783-1793.

LaPara, T. M., et al. (2011). "Tertiary-treated municipal wastewater is a significant point source of antibiotic resistance genes into Duluth-Superior Harbor." Environmental science & technology **45**(22): 9543-9549.

Le, T.-H., et al. (2018). "Removal of antibiotic residues, antibiotic resistant bacteria and antibiotic resistance genes in municipal wastewater by membrane bioreactor systems." Water research **145**: 498-508.

Lee, D.-Y., et al. (2006). "Detection of bacterial pathogens in municipal wastewater using an oligonucleotide microarray and real-time quantitative PCR." Journal of microbiological methods **65**(3): 453-467.

Lerminiaux, N. A. and A. D. Cameron (2019). "Horizontal transfer of antibiotic resistance genes in clinical environments." Canadian journal of microbiology **65**(1): 34-44.

Lew, B., et al. (2009). "Anaerobic membrane bioreactor (AnMBR) for domestic wastewater treatment." Desalination **243**(1-3): 251-257.

Li, Z.-H., et al. (2019). "Mitigated membrane fouling and enhanced removal of extracellular antibiotic resistance genes from wastewater effluent via an integrated pre-coagulation and microfiltration process." Water research **159**: 145-152.

Li, Z.-H., et al. (2021). "Evaluating the effect of gradient applied voltages on antibiotic resistance genes proliferation and biogas production in anaerobic electrochemical membrane bioreactor." Journal of hazardous materials **416**: 125865.

Liu, S.-S., et al. (2018). "Chlorine disinfection increases both intracellular and extracellular antibiotic resistance genes in a full-scale wastewater treatment plant." Water research **136**: 131-136.

Masco, L., et al. (2006). "Antimicrobial susceptibility of Bifidobacterium strains from humans, animals and probiotic products." Journal of Antimicrobial Chemotherapy **58**(1): 85-94.

Meiramkulova, K., et al. (2021). "Performance of a combined treatment approach on the elimination of microbes from poultry slaughterhouse wastewater." Sustainability **13**(6): 3467.

Miller, J. H., et al. (2016). "Survival of antibiotic resistant bacteria and horizontal gene transfer control antibiotic resistance gene content in anaerobic digesters." Frontiers in microbiology **7**: 263.

Naas, T., et al. (2011). "Real-time PCR for detection of NDM-1 carbapenemase genes from spiked stool samples." Antimicrobial Agents and Chemotherapy **55**(9): 4038-4043.

Neudorf, K. D., et al. (2017). "Antibiotic resistance genes in municipal wastewater treatment systems and receiving waters in Arctic Canada." Science of the Total Environment **598**: 1085-1094.

Nikolich, M. P., et al. (1994). "Evidence for natural horizontal transfer of tetQ between bacteria that normally colonize humans and bacteria that normally colonize livestock." Applied and environmental microbiology **60**(9): 3255-3260.

Noor, Z. Z., et al. (2021). "A review of bacterial antibiotic resistance genes and their removal strategies from wastewater." Current Pollution Reports **7**(4): 494-509.

O'Malley, K., et al. (2022). "An Extraction Method to Quantify the Fraction of Extracellular and Intracellular Antibiotic Resistance Genes in Aquatic Environments." Journal of Environmental Engineering **148**(5).

Osińska, A., et al. (2017). "The prevalence and characterization of antibiotic-resistant and virulent Escherichia coli strains in the municipal wastewater system and their environmental fate." Science of the Total Environment **577**: 367-375.

Ozgun, H., et al. (2013). "A review of anaerobic membrane bioreactors for municipal wastewater treatment: integration options, limitations and expectations." Separation and Purification Technology **118**: 89-104.

Pei, R., et al. (2006). "Effect of river landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG)." Water research **40**(12): 2427-2435.

Rafraf, I. D., et al. (2016). "Abundance of antibiotic resistance genes in five municipal wastewater treatment plants in the Monastir Governorate, Tunisia." Environmental pollution **219**: 353-358.

Reygaert, W. C. (2018). "An overview of the antimicrobial resistance mechanisms of bacteria." AIMS microbiology **4**(3): 482.

Ritter, W. (2021). "State regulations and guidelines for wastewater reuse for irrigation in the US." Water **13**(20): 2818.

Robles, Á., et al. (2021). "Anaerobic membrane bioreactors for resource recovery from municipal wastewater: a comprehensive review of recent advances." Environmental Science: Water Research & Technology.

Savin, M., et al. (2021). "Antibiotic-resistant bacteria, antibiotic resistance genes, and antibiotic residues in wastewater from a poultry slaughterhouse after conventional and advanced treatments." Scientific Reports **11**(1): 1-11.

Schlüter, A., et al. (2007). "Genomics of IncP-1 antibiotic resistance plasmids isolated from wastewater treatment plants provides evidence for a widely accessible drug resistance gene pool." FEMS microbiology reviews **31**(4): 449-477.

Shannon, K., et al. (2007). "Application of real-time quantitative PCR for the detection of selected bacterial pathogens during municipal wastewater treatment." Science of the Total Environment **382**(1): 121-129.

Sharma, V. K., et al. (2016). "A review of the influence of treatment strategies on antibiotic resistant bacteria and antibiotic resistance genes." Chemosphere **150**: 702-714.

Shen, Y., et al. (2019). "Degradation of antibiotics and antibiotic resistance genes in fermentation residues by ionizing radiation: a new insight into a sustainable management of antibiotic fermentative residuals." Journal of Environmental Management **232**: 171-178.

Shi, P., et al. (2013). "Metagenomic insights into chlorination effects on microbial antibiotic resistance in drinking water." Water research **47**(1): 111-120.

Silva, J. C., et al. (2004). "Factors that affect the horizontal transfer of transposable elements." Current issues in molecular biology **6**(1): 57-72.

Smith, A. L., et al. (2014). "Navigating wastewater energy recovery strategies: a life cycle comparison of anaerobic membrane bioreactor and conventional treatment systems with anaerobic digestion." Environmental science & technology **48**(10): 5972-5981.

Sui, Q., et al. (2019). "Fates of intracellular and extracellular antibiotic resistance genes and microbial community structures in typical swine wastewater treatment processes." Environment international **133**: 105183.

Szczepanowski, R., et al. (2009). "Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics." Microbiology **155**(7): 2306-2319.

Tong, H., et al. (2018). "High carriage rate of mcr-1 and antimicrobial resistance profiles of mcr-1-positive Escherichia coli isolates in swine faecal samples collected from eighteen provinces in China." Veterinary microbiology **225**: 53-57.

Van Hoek, A. H., et al. (2011). "Acquired antibiotic resistance genes: an overview." Frontiers in microbiology **2**: 203.

Viswanathan, V. (2014). Off-label abuse of antibiotics by bacteria. Gut Microbes. 2014; 5 (1): 3–4.

Von Wintersdorff, C. J., et al. (2016). "Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer." Frontiers in microbiology **7**: 173.

Vrouwenvelder, J., et al. (2010). "Integrated approach for biofouling control." Water science and technology **62**(11): 2477-2490.

Wang, J. and X. Chen (2022). "Removal of antibiotic resistance genes (ARGs) in various wastewater treatment processes: An overview." Critical Reviews in Environmental Science and Technology **52**(4): 571-630.

Wong, K., et al. (2009). "Removal of viruses and indicators by anaerobic membrane bioreactor treating animal waste." Journal of environmental quality **38**(4): 1694-1699.

Wu, B. and J. Kim (2020). "Anaerobic Membrane Bioreactors for Nonpotable Water Reuse and Energy Recovery." Journal of Environmental Engineering **146**(2): 03119002.

Yamamoto, Y., et al. (2022). "Horizontal transfer of a plasmid possessing mcr-1 marked with a single nucleotide mutation between Escherichia coli isolates from community residents." BMC Research Notes **15**(1): 1-6.

Zarei-Baygi, A., et al. (2019). "Evaluating Antibiotic Resistance Gene Correlations with Antibiotic Exposure Conditions in Anaerobic Membrane Bioreactors." Environmental science & technology **53**(7): 3599-3609.

Zarei-Baygi, A., et al. (2020). "Microbial community and antibiotic resistance profiles of biomass and effluent are distinctly affected by antibiotic addition to an anaerobic membrane bioreactor." Environmental Science: Water Research & Technology **6**(3): 724-736.

Zarei-Baygi, A. and A. L. Smith (2021). "Intracellular versus extracellular antibiotic resistance genes in the environment: Prevalence, horizontal transfer, and mitigation strategies." Bioresource technology **319**: 124181.

Zarei-Baygi, A., et al. (2020). "Membrane fouling inversely impacts intracellular and extracellular antibiotic resistance gene abundances in the effluent of an anaerobic membrane bioreactor." Environmental science & technology **54**(19): 12742-12751.

Zhang, J., et al. (2018). "Deciphering the factors influencing the discrepant fate of antibiotic resistance genes in sludge and water phases during municipal wastewater treatment." Bioresource technology **265**: 310-319.

Appendix A

Theoretical Biogas Production

$$CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O$$

The above stoichiometric equation was used for the maximum theoretical methane production

Thus, 16 g/mol + 64 g/mol → 44 g/mol + 36 g/mol

Thus, 0.25 g.CH_4 for each gram COD consumed.

Methane content normalization to CH_4 and CO_2:

$$CH_4(\%) = \frac{CH_4(\%)}{CH_4(\%) + CO_2(\%)} * 100.$$

Thereafter, actual methane volume was calculated based on the following formula: $V_{CH4} = V_{biogas} * \frac{CH4(\%)}{100}$, where $CH_4(\%)$ is the percentage of methane volume, V_{biogas} is the biogas volume recorded daily in L/day, and V_{CH4} is the actual daily methane volume in L/day.

Theoretical methane volume:

$$V_{CH_4\ Theoretical} = (V_{Influent} \times COD_{Influent}) \times COD_{Removal} \times Conversion\ rate_{COD\ to\ CH_4}$$

Where:

$V_{CH_4\ Theoretical}$ represent the expect daily methane volume (L/day)

$V_{Influent}$ represent the expect daily volume of influent fed to the reactor (L/day)

$COD_{Influent}$ represent the influent COD (mg/L)

$Conversion\ rate_{COD\ to\ CH4}$ is equal to 0.25 g CH_4 / g (appendix A)

The COD removed was then converted to L_{CH4}/g.COD by using the Ideal Gas Law formula:

$$Conversion\ rate_{COD\ to\ CH4} == \frac{0.25}{Mw} * R * T.$$

With R being the universal gas constant = 0.0821 L.atm/mol.k

T representing the ambient temperature in kelvin 298 K

M_w (methane) = 16.04 g/mol

Oversaturation ratio:

Oversaturation ratio = $\frac{V_{Effluent\ CH_4}\ calculated}{V_{Effluent\ CH_4}\ theoretical}$, where:

$$V_{Effluent\ CH_4}\ calculated\ \left(\frac{mL}{L}\right) = \frac{V_{Flask\ headspace}(mL) *\ CH_4(\%)}{V_{effluent\ in\ the\ flask}(mL)} * 1000\ (\frac{mL}{L})$$

$$V_{Effluent\ CH_4}\ theoretical\ \left(\frac{mL}{L}\right) = \frac{V_{CH4\ excpected\ in\ membrane\ effluwnt}(mL/day)}{V_{emembrane\ effluent\ collected}(L/day)} * 1000$$

$V_{CH4\ excpected\ in\ membrane\ effluwnt}(mL/day)$

$$= \frac{(K_H *\ CH_4\ percentage\ in\ headspace *\ 10^{-6}) * R * T}{M_w}$$

In which:

K_H is Henry's law constant representing the methane solubility in water at 35°C = 16.5 mg/L

R and T are the gas constant and absolute ambient temperature in kelvin respectively previously defined.

Purified genes concentrations

For the calculation of the total number of copies for the purified genes, the following formula was used:

$$Number\ of\ copies = \frac{Total\ concentration\ \times Avogadro's\ number}{Length\ \times 10^9 \times 650}$$

Where,

Total concentration is the total concentration of the purified gene per nanodrop (ng/uL)

Avogadro's Number = $6.023*10^{23}$

Length is the corresponding gene length (base pairs)

650 is Dalton's base pair weight

Appendix B

Gene	R^2	amplification efficiency	amplification factor
blaTEM	0.9978	91%	1.91
tetW	0.9995	88%	1.88
intl1	0.9879	102%	2.01
tetQ	0.9987	87%	1.87
sul1	0.9998	93%	1.93
tetA	0.9992	86%	1.86
ampC	0.9963	97%	1.97
tetC	0.9996	89%	1.89
mcr1	0.9997	92%	1.92
ereA	0.9995	80%	1.8

Table 3 qPCR target genes amplification efficiency

Amplification efficiency = $10^{-1/slope} - 1$

Where the slope represents the corresponding gene standard curve slope

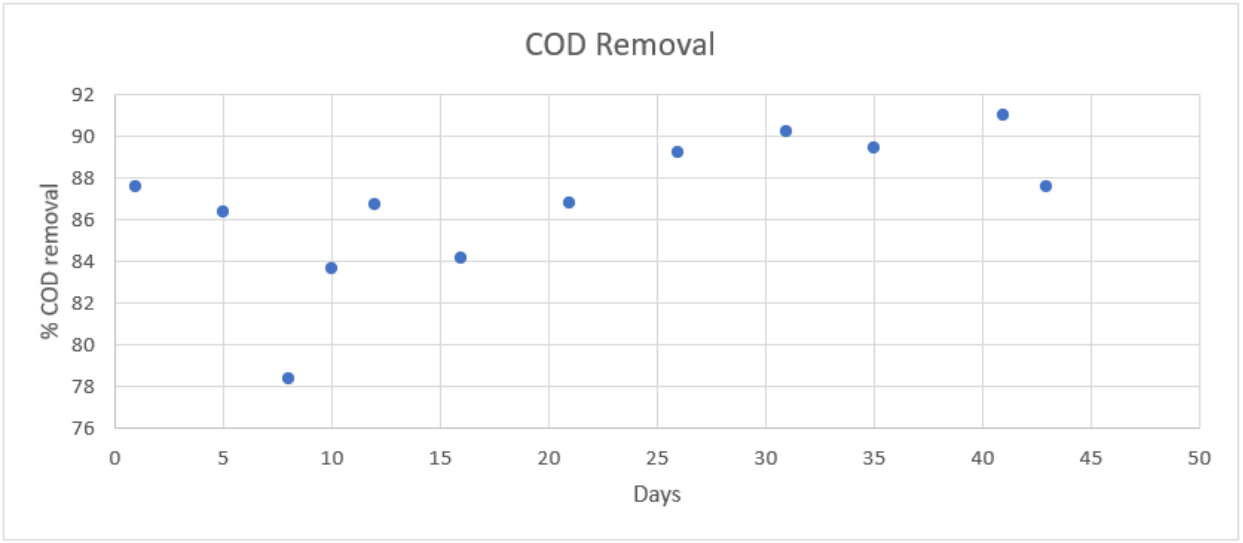

Table 4 Percentage COD removal throughout the experiment

DNA concentration normalization:

For influent and effluent:

$$\text{Copies/mL} = \frac{\text{Copies obtained by qPCR} \times 100\ \text{uL}}{\text{filtrated volume}}$$

Where,

100 uL represents the elution volume in the final step of the DNA extraction kit used and thus the volume of DNA obtained

Filtrate volume is the volume of influent or effluent used for the filtration

For sludge biomass:

$$\text{Copies/g. vss} = \frac{\text{Copies obtained by qPCR} \times 100\ \text{uL}}{\text{g.VSS}}$$

Where,

g.VSS = g.VSS/L * 0.002 L

Where,

g.VSS/L is the VSS present in the reactor at the sampling time

0.002 is the volume of sludge biomass collected (2 mL)

For membranes:

$$\text{Copies/cm}^2 = \frac{\text{Copies obtained by qPCR} \times 100\ \text{uL}}{\text{Membrane area (cm}^2\text{)}}$$

Where membrane area is the area of the corresponding membrane, calculated using the following formula:

$$\text{Membrane area (cm}^2\text{)} = \frac{\text{Membrane width} \times \text{Membrane length}}{\text{Membrane fraction}}$$

Where the membrane fraction is the fraction used to extract the biomass on the membrane (1/4 in this experiment)

For rpoB:

The same normalization equation as before for each biomass type, but with rpoB copies obtained from qPCR on the same samples, but targeting the rpoB gene

pGEM 3-z and extracellular DNA recovery

$$\text{pGEM 3-z recovery percentage} = \frac{\text{obtained copies by qPCR for each eDNA sample}}{\text{obtained copies by qPCR for aliquot}} \times 100$$

Where the obtained copies for aliquot is the number of copies obtained by qPCR for the aliquot used for spiking of the samples

$$\text{eDNA concentration normalized to plasmid} = \frac{\text{Normalized eDNA concentration } (\frac{\text{copies}}{\text{mL}})}{\text{pGEM recovery percentage}}$$