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Investigating the Fate of Tetracycline in an Anaerobic Membrane Bioreactor and Its Effects on Antibiotic Resistance Gene Proliferation from Different Membrane Types

By

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DEDICATION

I dedicate this work to Hadi, my supportive life partner who believed in me, unleashed the best of me, and supported me through every step of the way.
To my loving parents, brother, sister, and friends, you've been my best cheerleaders, and I thank you from the bottom of my heart for your words of encouragement and push for tenacity.

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Investigating the Fate of Tetracycline in an Anaerobic Membrane Bioreactor and Its Effects on Antibiotic Resistance Gene Proliferation from Different Membrane Types

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ABSTRACT

As the concern of fresh and clean water scarcity is raised worldwide, the necessity of water reuse stands out. In this sense, the AnMBR, an advanced wastewater treatment technology combining the advantages of the MBR and anaerobic processes, has gained much attention in the past years as it produces high quality effluent from an energy efficient mechanism. Even though the removal of emerging microbial contaminants generally and antibiotics specifically from wastewater has been studied, the reduction of ARGs and tetracycline, which is one of the most used antibiotics worldwide, hasn't been explicitly looked into using AnMBRs during treatment of real municipal wastewaters. Hence, this research, composed of a pre-tetracycline addition phase and a tetracycline addition phase, aimed to examine the effect of tetracycline continuously fed at a concentration of 300 µg/L on the performance of a lab-scale AnMBR as well as to evaluate the removal efficiency of tetracycline via the AnMBR system while looking into the attribution of each removal mechanism. It also studied the proliferation of iARGs and eARGs in the effluents of three externally connected membranes: two MFs and one UF. The results showed that a slight and quickly recoverable disruption was imposed by tetracycline on the system. Tetracycline was reduced at an efficiency > 91% for the three effluents with adsorption to sludge being the primary mechanism of removal even though notable degradation averaged to 38 % was recorded. The contribution of adsorption of tetracycline to the biofilm layer developed on each of the three membranes to removal was negligible. For ARGs, an increase in the abundance of *tet*-associated iARGs, with variation depending on the membrane pore size, was detected in the effluent, in opposition to *tet*-associated eARGs and non-*tet* ARGs which did not show an interpretable response to tetracycline.

Keywords: Pore size, Intracellular, Extracellular, Adsorption, Biodegradation

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LIST OF ABBREVIATIONS

AeMBR……. aerobic membrane bioreactor
AnMBR……. anaerobic membrane bioreactor
ARB ……. antibiotic resistant bacteria
ARG……..…….... antibiotic resistance gene
ASBR……... anaerobic sequencing batch reactor
CAS …….…… conventional activated sludge
CEC…… contaminant of emerging concern
COD…… chemical oxygen demand
CSTR……... continuous stirred tank reactor
DDD…… defined daily dose
eARG…….……. extracellular ARG
EDC…….…… endocrine disrupting trace pollutant
eDNA……...……... extracellular DNA
EGSB…… expanded granular sludge bed
EPS…… extracellular polymeric substances
HGT……...……. horizontal gene transfer
HPLC-UV…….…..…… high performance liquid chromatography – ultraviolet
HRAP……... high-rate algal pond
HRT…….……. hydraulic retention time
iARG……... intracellular ARG
iDNA…….…….……. intracellular DNA
MBR……..……. membrane bioreactor
MF……. microfiltration
MLSS …….……. mixed liquor suspended solids
MP……...…… micropollutants
MWCO……. molecular weight cut-off
NF……... nanofiltration
NOM……. natural organic matter
OLR ……. organic loading rate
OMP……. organic micropollutant

PhAC…… pharmaceutically active compound
PSA……..…… primary secondary amine
PVDF……..……... polyvinylidene fluoride
qPCR……..… quantitative polymerase chain reaction
RO……... reverse osmosis
SBR…….……. sequencing batch reactor
SMP …….……. soluble microbial products
SMPA…… staged multi-phase anaerobic
SPE…… solid phase extraction
SRT…….…… sludge retention time
SWW……. slaughterhouse wastewater
TMP……... transmembrane pressure
TrOC……..……. trace organic contaminant
TSS…… total suspended solids
UASB…….…. up-flow anaerobic sludge blanket
VFA…….……. volatile fatty acid
VSS……..…… volatile suspended solids
WWTP…….……. wastewater treatment plant

CHAPTER ONE

INTRODUCTION

1.1 Scarcity of Clean Fresh Water

Scarcity of clean fresh water has always been a major worldwide issue especially with the increased population growth and its associated repercussions. Even though water covers 71% of the earth surface, the vast majority (97.5%) of this totality is saline (Bhat, 2014). This leaves a relatively small fraction of freshwater for living things to fulfill their needs, starting from essentials like drinking, household needs, and agriculture and passing through developmental aspects of life like industry and power generation all the way to inconsequential matters like recreation. The consumption of this finite source would thus lead to: a) pollution which imposes a health threat for homo sapiens, fauna, and flora and b) overuse which risks drying up of freshwater bodies (lakes, streams, ponds, etc.). Being the cornerstone of all living things, this necessitates that people find a solution to ensure present and future provision of clean and safe freshwater, and this introduces the importance of water and wastewater treatment for reuse.

1.2 Wastewater Treatment Technologies

Through years, wastewater treatment and management have gone through a very tedious course to reach legitimate control yielding a satisfactory effluent product (Lofrano & Brown, 2010). This has been implemented through different levels of treatment under which various schemes, designs, and strategies fall; these levels can be categorized into

primary treatment, secondary treatment, advanced treatment, disinfection practices, and solid processing. Primary treatment is a physical kind of removal by which heavy solids settle by gravity; secondary treatment almost always involves a biological system where micro-organisms consume organics and produce water, carbon dioxide and/or methane yielding energy; advanced treatment could aim for the removal of nitrogen and phosphorus nutrients from the wastewater considering that the yielded effluent is not intended for irrigation purposes or removal of micro-pollutants and pathogens (Wenzel et al., 2008); and disinfection is applied to kill pathogens. As for solid processing, it targets the volatile solids and pathogens found in wastewater solids to be used as plant fertilizers or disposed in landfills (O'Kelly, 2005). Optimizations and advancements in secondary wastewater treatment in particular have been gaining a lot of attention for the past years especially that according to the EPA (1998), secondary treatment removes about 85% of the organics. In this sense, biological wastewater treatment was further divided into two general categories, either attached growth systems like trickling filters and rotating biological contactors (RBCs) in which microorganisms grow and attach on a media forming a biofilm layer, or suspended growth systems like activated sludge in which the free-floating microorganisms are combined with the substrate, i.e., waste (Ebrahimi & Najafpour, 2016). The selection of a wastewater treatment technology to adopt is a lengthy and intricate process that requires taking into consideration many aspects like the type and strength of the waste, required level of treatment, use of the effluent, land availability, cost, etc. (Kalbar et al., 2012; Rajasulochana & Prethy, 2016).

1.3 Biological Wastewater Treatment and Anaerobic Digestion

Another norm based on which biological wastewater treatment could be categorized is the presence or absence of oxygen and the types of microorganisms that are nourished

accordingly (Mittal, 2011). Aerobic systems involve oxygen supply for aerobic bacteria, i.e., aerobes, to utilize organics and produce water, carbon dioxide, and biomass. On the other hand, anaerobic systems do not involve oxygen supply, so anaerobic bacteria, i.e. anaerobes, thrive producing carbon dioxide, methane, and biomass as well. Even though there are several benefits for aerobic systems in comparison to anaerobic systems such as obtaining enhanced nutrient removal and not releasing unpleasant odors as there is no production of biogas especially hydrogen sulfide as in anaerobic (Anijiofor Sandra et al., (2017), anaerobic systems still present more substantial advantages. They are simpler, cheaper, produce small quantities of de-waterable sludge, occupy smaller space, and they not only require less energy uptake but also produce energy in the form of biogas that can be exploited for useful means like electricity generation (Anijiofor Sandra et al., 2017; Khan et al., 2011). This is besides the significant removal of contaminants for influents of high COD (> 4000 mg/L) at high or low organic loading rates. Anaerobic digestion, despite being one of the most old-established water and wastewater stabilization technologies and dating back to the nineteenth century, has gained more popularity and interest in the past years; more efforts have been assigned to revamp and optimize this technology in intentions of obtaining a more cost-effective means of treatment that outweighs conventional wastewater schemes in terms of uncoupling the hydraulic retention time (HRT) and solids retention time (SRT) for 'high-rate' reactors (Van Lier et al., 2001).

1.4 Anaerobic Membrane Bioreactor Technology

One of the advanced wastewater treatment technologies, ramifying from anaerobic digestion, that have been gaining great recognition for the past years is the membrane bioreactor (MBR). The membrane bioreactor, in simple terms, is the pairing of two processes together which are the biological treatment process, i.e., bacterial digestion of organics, and

separation or retention of suspended solids via a membrane to obtain particulate-free effluent (Sutherland, 2010). Although MBRs are considered among the modern wastewater treatment systems, the idea of coupling microbial digestion with a very fine filter was first established by Dorr Oliver in the mid-1960s; however, not until 1989 did the membrane bioreactor technique quite evolve. In 2005, the market value of the MBR technology was estimated to $217 million, increasing at a yearly growth rate of 10.9% on average which is notably faster than other developed wastewater treatment approaches (Hanft, 2006). However, in recent past distinctive attention has been given to the anaerobic operation of the MBR due to the outstanding advantages of it in comparison to aerobic operation. To this day, seeking enhancements and revamps in the anaerobic membrane bioreactor (AnMBR) system and surmounting the challenges it imposes has been prompted by environmentalists and engineers in hopes of getting the best out of this promising breakthrough.

1.5 Antibiotic Presence in Wastewater

Contaminants of concern (CECs), also called micropollutants (MPs), are trace organic contaminants (TrOCs) that originate from different sources and end up in the wastewater and consequently in the aquatic environment if not treated properly before discharge (Barbosa et al., 2016; Kanaujiya et al., 2019; Matamoros & Bayona, 2006; Wanda et al., 2017). These micropollutants could emanate from wastewater of household, municipal, industrial, or agricultural origin; they could even come from hospital wastewater, landfill leachate, and wastewater emerging from slaughterhouses and livestock husbandries (Mompelat et al., 2009). Even though their concentration ranges between several nanograms per liter to few micrograms per liter, they have a major and severe effect on the environment if accumulated and spread. Micropollutants include a wide range of compounds that could be natural (e.g., hormones such as natural estrogens) or anthropogenic such as

pharmaceuticals (antibiotics, steroid hormones, etc.), personal care products, pesticides, industrial and chemical compounds, and polycyclic aromatic hydrocarbons. In the recent 10 years, the consumption of antibiotics worldwide has increased rapidly (Borghi & Palma, 2014), and because a significant fraction (ranging from 25% to 75%) of these antibiotics ingested by humans and animals gets released unmetabolized, their presence can no longer be overlooked (Khan and Ongerth, 2004; Rivas et al., 2011; Watkinson et al., 2009). Tetracycline is one of these antibiotic classes that is given greater attention to on the grounds that it imposes critical environmental problems including ecological perils and human health threats (Daghrir & Drogui, 2013).

1.6 Antibiotic Resistance: A Critical Health Threat

The excessive and uncontrolled production and use of antibiotics in the past few decades has induced widespread antibiotic resistance in bacteria (Neu, 1992). In simple terms, antibiotic resistance is when the bacteria becomes unresponsive to the explicit effect of antibiotics: prompting cell death (bactericidal antibiotics) or restraining cell growth (bacteriostatic antibiotics) (Kohanski et al., 2010). Bacterial infections could be fatal but alleviated and treated by appropriate antibiotics; however, with the escalation of antibiotic resistance, bacteria get acclimated and don't get affected upon exposure to a specific or group of antibiotics. The main driver that exacerbates antibiotic resistance is horizontal gene transfer (HGT) during which the resistance gene or genes are transferred to bacteria through various means (Von Wintersdorff, 2016). The discharge of different types of wastewaters that are likely to contain antibiotics into the environment without prior antibiotic-targeted treatment causes higher release of antibiotics and consequently more exposure provoking antimicrobial resistance. Although the dissemination of antibiotic resistance genes (ARGs) is inevitable and resistance developing in a specific region might easily get transmitted

globally (Cars & Nordberg, 2005), the human role in this sense would involve controlling the use of antibiotics especially in hospitals to obtain wastewater of lower antibiotic concentration, in addition to lowering the antibiotic concentration further through appropriate treatment systems.

CHAPTER TWO

LITERATURE REVIEW

2.1 Mainstream Anaerobic Treatment in Comparison to Conventional Aerobic Processes: Benefits, Challenges, and Limitations

Mainstream anaerobic treatment has developed as an auspicious and favorable alternative to conventional aerobic wastewater treatment systems in light of the noteworthy preferences it has demonstrated. To begin with, anaerobic treatment allows the decomposition of soluble organic compounds found in the wastewater ultimately converting them into biogas i.e., CH_4 and CO_2. This is attained through a series of four processes: hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Kanafin et al., 2021). Hydrolysis allows the breaking down of organic macromolecules, by the action of enzymes released by microorganisms, into compounds of lower molecular weight: proteins into amino acids, lipids into fatty acids and glycerol, and polysaccharides into monosaccharides (glucose, fructose, and galactose). Acidogenic bacteria then metabolize the products of hydrolysis to short chain fatty acids (lactic acid, propionic acid, butyric acid), carbon dioxide, hydrogen, and ethanol. Acetogenesis occurs afterwards during which anaerobic bacteria transform the intermediate products of acidogenesis into carbon dioxide, hydrogen, and acetic acid. Finally, methanogens yield CH_4 and CO_2.

Out of the systems that fall under mainstream anaerobic treatment technologies, the upflow anaerobic sludge blanket (UASB) reactor, expanded granular sludge bed (EGSB) reactor, staged multi-phase anaerobic (SMPA) reactor, anaerobic sequencing batch reactor

(ASBR), and anaerobic membrane bioreactor (AnMBR), which is the main focus of the presented work, are among the ones which prevail in terms of interest.

The UASB was introduced in the early 70s, and the efficiency of it depends on anaerobic biological processes which take place at the bottom of the reactor due to the growth and accumulation of a dense sludge bed (Seghezzo et al., 1998). Intents of optimizing the UASB reactor led to the rise of the EGSB reactor which disposes dead zones caused by inadequate internal mixing and enhances the contact between sludge and wastewater. The difference mainly is that EGSB adopts a taller and narrower reactor configuration and requires effluent recirculation in addition to using granular sludge at high upflow velocities.

Regarding the SMPA reactor, it involves the operation of multiple digestors in a chain to optimize the conditions needed for each of the treatment process reactions (Neba et al., 2019). As for the ASBR, it engages a cycle composed of four steps during which the food to microorganisms ratio is varied in due course; these steps are: feed, reaction, biomass flocculation & settling, and discharge (Akil & Jayanthi, 2012).

Speaking about the AnMBR, it has been tested for various types of wastewaters of COD values varying over a wide range. These include synthetic wastewater, food processing wastewater of different origins, non-food processing industrial wastewater, high-solids-content waste stream (sludge, manure, etc.), and municipal wastewater (Liao et al., 2006). Predominantly, promising yet varying results were obtained depending on characteristics of wastewater being treated and the operating conditions adopted such as the organic loading rate (OLR) and the hydraulic retention time (HRT).

Regardless of the fact that the phenomenon of anaerobic digestion was first reported four centuries ago and first employed for wastewater treatment in 1881, it was not until a few decades back that it has been subjected to intent scientific scrutiny (Abbasi et al., 2012b;

McCarty et al., 1982) particularly after the precipitous boost in energy prices in the 1970s (Seghezzo et al., 1998). The reason for that goes to the substantial advantages that anaerobic digestion has demonstrated in comparison to aerobic processes, the foremost of which is energy conservation and recovery (Kleerebezem & Macarie, 2003). This is established by reducing the need of large energy supply for mechanical mixing for aeration in aerobic systems, as well as producing methane-rich biogas which is a resource of concernment for developing countries mostly because of the shortage and high expense of energy supply (Abbasi et al., 2012b). Also, anaerobic digestion, due to the fact that anaerobic bacteria have slow growth rates, tends to produce around 10% of the biomass produced in aerobic systems which is incorporated with less costly subsequent sludge handling (Kleerebezem & Macarie, 2003). More than that, in case the wastewater to be treated happened to contain low amounts of nutrients necessary for the growth of biomass, like the case of chemical and petrochemical wastewater, this would necessitate adding nutrients (N, P, S, etc.) per quantity of biomass available to trigger their growth and ensure competence of the system in hand. When less biomass is produced, as in case of anaerobic digestion, less nutrients would need to be added meaning less cost as well. It is also important to note that anaerobic sludge could be used as an inoculum for starting up new reactors considering that it can be sustained for a significant period of time without decline in biological activity (Akunna, 2018). Beside the relative simplicity of construction and operation of anaerobic reactors and their flexibility of application in terms of scale, anaerobic wastewater treatment systems have a smaller footprint especially when high loading rates are contemplated.

In spite of the numerous advantages that induced shifting research focus towards energy-saving substitutes like anaerobic systems and pared the attractiveness of aerobic processes, mainstream anaerobic treatment incorporates notable disadvantages and experiences some challenges. To begin with, anaerobic digestion is accompanied by the

production and release of hydrogen sulfide which has a bad odor especially when the wastewater to be treated contains high protein and sulfate contents; raising the need for introduction of a component for biogas handling (Akunna, 2018; Syed et al., 2006). Also, the slow growth rate of methanogens makes the start-up phase of anaerobic systems longer than aerobic ones. Even though anaerobic treatment systems have demonstrated high efficiency in terms of COD removal, even at critical conditions of low temperature and high loading rates, they exhibit low pathogen and nutrient removal which necessitates the incorporation of a post-treatment unit. For micropollutants such as antibiotics, anaerobic treatment processes have displayed competence of varying efficiency in removal of antibiotics depending on their type and concentration besides the operating conditions of the treatment system. Nonetheless, most previous studies have looked into the removal of antibiotics under aerobic conditions instead (Cheng et al., 2018a). Another underlying challenge is the high sensitivity of anaerobic bacteria to some environmental conditions such as temperature, pH and toxic compounds presence and concentration (Seghezzo et al., 1998). Thence, mainstream anaerobic treatment has been a prevalent focus of wastewater research for the past years with thorough effort put into optimizing the process and overcoming its challenges.

2.2 Shift of Focus from Conventional Activated Sludge Systems to Anaerobic Membrane Bioreactors

2.2.1 Conventional Activated Sludge System Vs. Aerobic Membrane Bioreactor

A membrane bioreactor (MBR) embodies the conventional activated sludge (CAS) process but excludes the secondary clarifier and tertiary treatment and incorporates in conjunction with it an additional element which is physical membrane separation (Melin et

al., 2006). The benefits of MBRs promoting their adoption over conventional activated sludge process include smaller space requirement as a result of the omission of posterior units as well as higher biomass concentration and loading rate (Gander et al., 2000; Marrot et al., 2004; Melin et al., 2006). A dense biomass concentration of 20 g/L (Jefferson et al., 2000) and even 30 g/L (Yamamoto et al., 1988) was utilized for the MBR systems, which is four or more times higher than that utilized by conventional treatment schemes (less than 5 g/L) (Marrot et al., 2004). Aside from that, they produce less sludge by a factor of 2 to 3, yield better effluent quality ensured by membrane separation and high SRT which increases sludge concentration and the organic load, and demonstrate less responsiveness or affection to contaminant peaks. According to Huang et al. (2001) who studied the effect of varying SRT on the efficiency of treatment of each of the MBR and conventional bioreactor, the MBR maintained a COD removal of 90% whereas the conventional bioreactor recorded 70 to 80% COD removal with a small decrease at SRTs of 5 to 10 days. This accentuates the performance of MBR being superior and more versatile to than that of conventional activated sludge systems, especially for COD and dissolved organic carbon removal (Ciardelli et al., 2001; Çiçek et al., 1999) as well as solid suspension separation according to Dufresne et al. Moreover, looking into trace pollutants and microorganisms, the MBR has been observed to surpass the CAS in terms of removal of endocrine disrupting trace pollutant (EDCs) which exhibit estrogenic activity (Holbrook et al., 2004; Zühlke et al., 2003), in addition to removal of microbiological parameters particularly bacteriophages (5.88 vs. 1.31 log removal) and fecal coliforms (6.86 vs. 2.34 log removal) (Ueda & Horan, 2000). Many previous studies also highlighted that aerobic MBRs (AeMBRs) manifested better removal of organic micropollutants in comparison to CAS (Cirja et al., 2008; De Wever et al., 2007; Radjenovic´ et al., 2009; Zuehlke et al., 2006). Le at al. (2018) also identified that the MBR outperformed CAS in the removal of most of seventeen addressed

and detected antibiotics (trimethoprim, tetracycline, sulfamethazine, oxytetracycline, minocycline, meropenem, lincomycin, erythromycin-H20, ciprofloxacin, chlortetracycline, clarithromycin) as well as antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs).

Notwithstanding the diverse features that have made MBRs eclipse the conventional activated sludge processes, these advanced membrane-based techniques bring adversities that raise apprehension and require appropriate handling and/or maintenance. The first limiting factor is membrane fouling caused by the development of a layer or cake on the membrane and/or the penetration of particles into its pores (Marrot et al., 2004). If membrane fouling, which is evinced through increase in the transmembrane pressure, is not controlled, this leads to a decrease in the flux (Marrot et al., 2004; Melin et al., 2006). The phenomenon of membrane fouling is affected by multiple diverse factors such as the aeration rate, concentration of the mixed liquor suspended solids (MLSS), biomass characteristics, soluble microbial products (SMP), extracellular polymeric substances (EPS) particularly the carbohydrate and protein fraction of the biomass supernatant and fouling layer, presence of higher molecular weight compounds such as polyelectrolytes, and membrane type, chemical nature and module configuration in terms of pore size and surface morphology (Babu et al., 2001; Carroll, 2001; Hirose et al., 1996; Hwang & Lin, 2002; Le-Clech et al., 2006; Marrot et al., 2004; Melin et al., 2006; Vrijenhoek et al., 2001). Other elements of influence include hydrodynamic conditions such as the mode of operation (constant TMP or constant flux) and filtration time. Because numerous linked and unlinked aspects influence membrane fouling and because the pursuit of assigning fouling in MBRs to a single parameter seems futile, total eradication of fouling is not possible, at least when taking the economical aspect into consideration and disregarding costly means like

ozonation and sonolysis proposed by Prado et al. (2017). This prompts adopting practices that either delay fouling or handle it after happening such as washing and cleaning the membrane which also conspicuously increases the maintenance and operating costs (Le-Clech et al., 2006). Furthermore, specific practices involve increasing the aeration rate as per Melin et al. (2006) who pointed out that membrane fouling can be managed by increasing the air-flow rate if a provisional increase in permeate flux occurs. On the whole, the membrane bioreactor edges out the conventional activated sludge processes regardless of the fouling limitation it imposes.

2.2.2 Aerobic vs. Anaerobic Membrane Bioreactor

In light of the high energy costs imposed by AeMBR and the urge to economize MBR and develop a more efficient and environmentally friendly format of it, the AnMBR was introduced. Besides being energy efficient, the AnMBR is favorable when compared to AeMBR in terms of yielding higher effluent quality and less sludge production (Mei et al., 2016). AnMBR conjoins the advantages of anaerobic digestion and membrane technology. The collection of the methane released into the headspace of the the AnMBR and recovery of methane dissolved in the effluent constitute the energy account of the AnMBR not offered in AeMBR. Although only 35% of the combusted methane produced is converted to electricity, the remaining 65% is emanated as heat (about 35 °C) (EPA, 2007) which can be employed to ensure mesophilic conditions and accelerate the slow rate of proliferation of anaerobic bacteria especially in cold environmental conditions (Pretel et al., 2015). Other benefits of AnMBR over AeMBR include yielding higher removal rates for multiple antibiotic-type organic micropollutants (OMPs) such as sulfamethoxazole and trimethoprim and associated antibiotic resistance genes (*sul1* and *sul2*) as reported by Harb et al., 2016. Even though Liu et al. (2020) showed that AeMBR surpassed the AnMBR in removal of

various trace organic micropollutants (TrOCs), the AnMBR recorded higher removal for compounds containing nitrogen in their molecular structures (e.g., amitriptyline, carbamazepine, and atrazine). This brings on the particularity incorporated with the removal of emerging contaminants from wastewater using AnMBR and the need to inspect each micropollutant of interest exclusively.

One further noteworthy difference between AeMBRs and AnMBRs is at the level of membrane fouling. The general picture displays that AnMBR treatment usually confronts more serious membrane fouling problems; thus, constituting a critical obstacle that restricts their more widespread application (Lin et al., 2013). However, looking deeply into this, more could be said. Membrane fouling occurs due to the interaction between the membrane material and the constituents of sludge suspension. AnMBRs have demonstrated unique membrane fouling characteristics because of the substantial difference of the sludge suspension in AnMBR even though the membrane used in both AeMBR and AnMBR systems can typically be the same. Jeison & van Lier (2007) and Gao et al. (2011) noticed that during the long-term operation of a submerged AnMBR, the cake formation and consolidation, which could not be cleared away by the typical means like back-flush cycles or relaxation, comprise the preeminent mechanism of membrane fouling prevailing over internal pore fouling. On the other hand, Di Bella et al. (2007) declared that the cake layer developed in an AeMBR is generally removable. This brings to a conclusion that the variation of sludge properties between AeMBR and AnMBR, the cake layer formed in AnMBR has relatively lower removability than that in aerobic setting. This translates into an increase in the energy requirement for gas scouring which needs to be reduced if they are to be preferable over AeMBRs.

2.3 Micropollutants in Municipal Wastewater

2.3.1 Routes Leading to Micropollutant Presence in Municipal Wastewater

The continued intensification of consumption and thus production of pharmaceuticals, personal care products, surfactants, pesticides, and many other industrial chemicals has put the aquatic environment, and subsequently human health, at risk (Chavoshani et al., 2020; Tosun et al., 2020). This becomes of huge concern especially when these micropollutants (MPs) end up in the municipal wastewater stream destined to be treated in municipal wastewater treatment plants (WWTPs) which are not usually designed for their removal (Edokpayi et al., 2017; Iloms et al., 2020, Luo et al., 2014). This is the case for developing countries where hospital wastewater is often discharged into municipal wastewater streams (Pauwels & Verstraete, 2006; Timraz et al., 2017; Verlicchi et al., 2015). Also, the introduction of trace organic contaminants into municipal drains is a resultant of the discharge of industrial effluents into their system, like in case of Nigeria, particularly Lagos, and South Africa (Iloms et al., 2020; Ntuli, 2012; Siyanbola et al., 2011). It has been reported that even in some developed countries like Canada, Ontario specifically, several slaughterhouses tend to dump their wastewater into the municipal sewer stream after primary pretreatment which plays no role in contaminant removal (Bustillo-Lecompte et al., 2016).

Because most WWTPs are designed and operated to remove organics and macro pollutants only (Macedo et al., 2021), in one way or another, a fraction of these emerging contaminants of variable size and disparate ramifications gets released from the WWTP into rivers and streams. This makes WWTPs concentrated point sources of pollution (Daughton & Ternes, 1999; Musolff et al., 2008) and thus at odds with their deliberate objective of protecting the environment and forestalling the fallout of not doing so on the aquatic and

terrestrial ecosystems. So, over the course of time, contamination of the water body accrues as water flows and harbors other micropollutants from sundry sources aggravating the problem and magnifying its health-threatening consequences (Macedo et al., 2021). In 2012, around 143,000 MPs were enrolled in the European market, many of which would eventually get released into the water bodies (Das et al., 2017).

So, delving into the presence, fate, impact, and removal of the numerous micropollutants that would end up in wastewater and thus in the environment is arduous and ramified due to the specificity and uniqueness of each type within MPs, class within types, and compound within classes. Margot et al. (2015) reviewed and compiled the findings from around 50 papers to summarize the presence of a substantial group of micropollutants in municipal wastewater and their removal using conventional wastewater treatment systems. The study affirmed the idiosyncrasy of each contaminant. Luo et al. (2014) also compiled data from various former studies on the percentage removal of specific compounds via conventional WWTPs.

2.3.2 The Competence of Membrane Technology in Micropollutants Removal

Membrane technology, particularly nanofiltration (NF) and reverse osmosis (RO), also exhibits good efficiency in micropollutant removal although microfiltration (MF) and ultrafiltration (UF) which are of less 'tight' configuration could contribute to some removal; that is through the binding of OMPs to colloidal organic carbon retained by the membranes not direct retention of the OMPs as they are usually smaller than the pore size of the membrane (Lim et al., 2020; Sahar et al., 2011; Schäfer et al., 2011). Furthermore, the OMP elimination role played by the MF or UF membrane could be via charge repulsion, the rejection capacity of which is governed by the physicochemical properties of both the micropollutant and the membrane (Lim et al., 2020). This is because micropollutants

removal via membrane is not only due to their physical retention by the small pores of the membrane, but also adsorption on to the polymers that make up the membrane and interaction with the natural organic matter (NOM) naturally found in the wastewater and contributing to fouling (Jermann et al., 2009). Prior research also looked deeply into the removal of multiple micropollutants during membrane processes of different types and conditions (membrane material and transmembrane pressure) and treating different types of wastewaters (Jermann et al., 2009; Röhricht et al., 2009; Sahar et al., 2011; Yangali-Quintanilla et al., 2011).

2.3.2.1 The Status of MBRs for Micropollutants Removal

Speaking about MBRs, Radjenovic et al. (2009) affirmed that they can significantly remove a large assortment of micropollutants even compounds that are impervious to activated sludge systems. This can be explained by the retention of sludge onto which the compounds adsorb, extended SRT that prompts the development of more microbial communities spurring the degradation of further compounds, and the physical blockage served by the membrane configuration (Spring et al., 2007). The SRT of an MBR might in some cases be measured in months rather than days. For instance, an SRT of 30–60 days was adopted for the operation of a full-scale MBR in Porlock, UK treating 1900 m^3/day of municipal wastewater (Judd, 2006). On the other hand, the typical SRT of the CAS processes ranges from 3 to 15 days (Tchobanoglous et al., 2003). Radjenovic et al. (2009) also accentuated on MBRs overshadowing CAS processes by maintaining steadier and more significant removal of pharmaceutically active compounds (PhACs) and pointed out MBR sludge being less contaminated with PhACs in comparison to CAS which means it would impose a smaller environmental risk in case desorption occurs. Trinh et al. (2012) studied the removal of 48 micropollutants from various categories using a full-scale MBR and

determined above 90% removal for the majority of them and a removal in the range of 24-68% for 9 out of the 48 emerging compounds of interest (amitriptyline, carbamazepine, diazepam, diclofenac, fluoxetine, gemfibrozil, omeprazole, sulfamethoxazole and trimethoprim). Other studies that have drawn the same conclusion of MBR technology outperforming CAS processes for MPs elimination are Chen et al. (2008) and Sahar et al. (2011).

2.3.2.2 Mechanisms of Removal of OMPs in AnMBRs

However, the elimination of OMPs and having insights into its mechanisms in the anaerobic configuration of the MBRs haven't been thoroughly studied (Lim et al., 2020). Sorption and biodegradation have been identified as the primary mechanisms affecting OMP elimination in anaerobic systems generally and AnMBR specifically (Gonzalez-Gil et Al., 2018; Harb et al., 2019). Sorption is attained through electrostatic interactions which involve cation bridging and exchange or through hydrophobic interactions (Cheng et al., 2018a; Luo et al., 2014). For instance, lipophilic OMPs can cling to hydrophobic membranes while OMPs generally can be retained by charged membranes through electrostatic interaction (Alvarino et al., 2018). Biodegradation mainly involves the decomposition of OMPs by microbes; as for volatilization it is considered a fundamental mechanism of removal for semi-volatile or non-biodegradable compounds which is often not the case of estrogens and pharmaceuticals (Suárez et al., 2008). A study done by Monsalvo et al. (2014) involved examining the efficiency of elimination of a wide spectrum constituting of 38 OMPs using the AnMBR technology; 29 of which got removed at an efficiency below 50% leaving only 9 of highly significant removal efficiency exceeding 90%. The variation of degree of biodegradation among compounds was also inspected as well as the variation among different OMPs of the time required to get accustomed to the

AnMBR environment. Lim et al. (2020) also compiled information on the removal efficiency of various OMPs using multiple hybrid membrane bioreactor systems. In brief, regardless of the diversification of micropollutants and their properties, research has generalized some design and operational controls that could aid in improving OMPs removal in AnMBRs (increasing SRT and/or HRT, incorporating biomass carriers, increasing temperature for volatile OMPs, etc.).

2.4 Antibiotics in Municipal Wastewater

2.4.1 Antibiotics Presence in Municipal Wastewater

One of the most significant subclasses of emerging contaminants that have been detected in municipal wastewater and gained a great deal of research attention in recent years is antibiotics falling under pharmaceuticals (Hernández et al., 2007). In simple terms, antibiotics are medicines used to prevent and treat bacterial infections in humans and animals (Daughton & Ternes, 1999; Richardson et al., 2005; World Health Organization, 2020) either by killing bacteria or precluding them from multiplying and reproducing (Felson, 2021). While the term antibiotics covers a wide range of classes (eleven, characterized by chemical structure), Zhang & Li (2011) and Zhang (2016) noted that antibiotics falling under 6 classes are the ones that are often identified in wastewater influents and effluents as well as activated and digested sludges; these are β-lactams, sulfonamides, quinolones, tetracyclines, macrolides, and others. The ‘others’ class basically groups thiamphenicol, choramphenicol, trimethoprim, lincomycin, and clindamycin (Díaz-Cruz & Barceló, 2005). Three major sources of these antibiotics are private households, hospitals, and industries (Giger et al., 2003; Wang et al., 2016) such as animal husbandry, slaughterhouses, and food production (Daughton & Ternes, 1999; Savin et al., 2021).

Collecting sales data from hospital pharmacies and retail from 71 countries, Van Boeckel et al. (2014) assessed the overall increase in antibiotic consumption between 2000 and 2010 which turned out to be equal to 35%, and they ascertained that Brazil, Russia, India, China, and South Africa contributed to 76% of this escalation. For household usage of antibiotics, Browne et al. (2021) evaluated, based on 209 surveys conducted between 2000 and 2018, a 46% increase in the global antibiotic consumption rate, from 9.8 defined daily doses (DDD) per 1000 population per day in 2000 to 14.3 DDD per 1000 population per day in 2018. Van Boeckel et al. (2015) also estimated the global antibiotic consumption in livestock to be 63,151 tons for the year 2010 taking into account 228 countries; they also anticipated a 67% increase in this amount by the year 2030 which would give rise to even more perilous environmental and health-related consequences.

However, it's important to note that the degree of consumption of an antibiotic alone doesn't rigorously signify the extent of its presence in municipal wastewater as the properties of the antibiotic also play a role in that. To illustrate, in spite of the attribution of β-lactams to the highest fraction of the total human antibiotic consumption, its existence was not often detected due to its unstable nature originating from the β-lactam ring being susceptible to hydrolysis (Christian et al., 2003; Färber, 2002; Kümmerer, 2009).

2.4.2 How Antibiotics End Up in The Environment

Once antibiotics are ingested by humans or animals, they get partially digested and absorbed and subsequently released in the urine and feces in both forms of the original unmetabolized molecules and metabolites which are hydroxylated, hydrolyzed, or conjugated structures of the parent molecule (Ikehata et al., 2006). For humans, Frade et al. (2014) specified that a fraction within the range of 30-90% of the antibiotic dose gets excreted 8-24 hours post ingestion. Likewise for livestock, the percentage of excretion of

different veterinary antibiotics quite varies; for instance, it is between 50-100% for tyolsin, 75% for chlortetracycline, and 90% for sulfamethazine (Kim et al., 2011).

The environmental exposure to veterinary antibiotics is not only via urine and feces discharge particularly, but generally through disposals of slaughterhouses. Slaughterhouse wastewater (SWW) is composed of paunch, feces, urine, blood, fat and lard, carcasses, undigested food, pharmaceuticals, and loose meat beside other stuff like lint, microbial pathogens, disinfectants, suspended material, and facility cleanings (Al-Mutairi et al., 2004; Bustillo-Lecompte & Mehrvar, 2015). The fat, lard, carcasses, and loose meat thus contribute to antibiotic release as multiple studies pointed out the presence of antibiotic residues in animal tissues (Barling & Selkon, 1978; Leitner et al., 2001; Mohamed et al., 2011; Mulders et al., 1989). The fallout of antibiotics occurrence in slaughterhouse wastewater is amplified when it is disposed into the sewers and allowed to mix with municipal wastewater treated in municipal WWTPs.

Aside from wastewater and WWTPs being a critical provenance for antibiotics release into surface water, antibiotics are also directly addressed into surface waters to treat and prevent diseases occurring in aquaculture. This is the case of China, for example, which is the largest producer and exporter of aquatic products (Liu et al., 2017; Shao et al., 2021). Therefore, the environment is susceptible to being jeopardized by antibiotics through various routes and approaches summarized in Figure 1 (Liao et al., 2021).

Figure 1 – Migration of Antibiotics and Their Main Removal Routes in WWTPs (Liao et al., 2021)

2.4.3 Impact of Antibiotic Presence in Municipal Wastewater on the Environment: Antibiotic Resistance

The substantial increase in the synthesis and consumption of antibiotics in different domestic, health, and industrial sectors for the past few decades has amplified the concern of development and accumulation of antibiotic resistant genes (ARGs) and antibiotic resistant bacteria (ARB). According to the WHO (2020), antibiotic resistance occurs when bacteria change in response to the use of antibiotics. Bacteria develop or engulf and carry ARGs, which encode a resistance mechanism, and become ungoverned nor killed by antibiotics; instead, they'd persist and even reproduce in the presence of an antibiotic or a group of antibiotics (MacGowan & Macnaughton, 2017). This happens when plasmids and/or transposons which are mobile genetic elements carry resistance genes and get transferred to a bacterium by horizontal gene transfer via conjugation, transduction, or transformation. Conjugation is when a bacterium possessing a plasmid holding ARG(s) replicates this plasmid and passes it on to another bacterium through direct cell-to-cell

contact. As for transduction, it defines the process during which a bacteriophage, which is a virus that attacks bacteria and replicates in the bacterial cell, integrates a fragment of bacterial DNA in the assembled viral particle then delivers it to the next bacterial cell that the virus gets passed on to. Meanwhile, the uptake of DNA freely floating in the environment is referred to as transformation. In short, in one way or another, the over-prescription of antibiotics especially in countries having no standard treatment guidelines, is risking the ability to treat prevalent infectious diseases such as pneumonia, tuberculosis, blood poisoning, gonorrhoea, and foodborne diseases (World Health Organization, 2020).

Nowadays, a minimum of 700 thousand people aredying because of drug-resistant diseases each year; over 230 thousand of them die as a result of multidrug-resistant tuberculosis (World Health Organization, 2019). Besides its fatal health consequences (increased morbidity, mortality, length of hospital stay, microbial transmission, etc.), antimicrobial resistance has economic implications (MacGowan & Macnaughton, 2017). The European Centre for Disease Prevention and Control (2017) also evaluated that each year, around 25,000 people in Europe die as a result of infections caught from hospitals and caused by resistant bacteria translating into a loss of €1.5 billion to healthcare and society (e.g., productivity). Therefore, antimicrobial resistance is a universal problem which calls for international action to preserve currently available antibiotics and establish new ones.

2.5 Tetracyclines: Broad Spectrum Antibiotics

2.5.1 Tetracycline Uses and Subsequent Presence in Municipal Wastewater

Tetracyclines, discovered in the 1940s, are a family of broad-spectrum antibiotics used in various sectors for multiple purposes: veterinary use, human therapy, and agricultural practices (Chopra & Roberts, 2001; Shutter & Akhondi, 2019). These

antibiotics enter into bacterial cells through passive diffusion and interfere with protein synthesis or destroy the membrane by binding with aa-tRNA binding site A on the 30S subunit of bacterial ribosome consequently hindering bacterial growth (Figure 2) (Liao et al., 2021; Schnappinger & Hillen, 1996).

Figure 2 – Mechanism of Tetracycline Inhibiting Protein Synthesis (Liao et al., 2021)

Cell-m

The concentrations of emerging organic micropollutants in WWTPs are governed by multiple variables such as the dose and pattern of pharmaceuticals consumption, the influent load etc., which would probably differ within one region or country and from one country to another (Radjenović et al., 2009). However, Gurung et al. (2019) evaluated the concentration of tetracycline in municipal wastewater generally to be < 1000 ng/L, which is mostly the case in municipal wastewater (Guerra et al., 2014). Nevertheless, the diversified

applications of tetracyclines have ramified the means of their emission into the sewers then WWTPs and eventually into the environment.

To begin with, veterinary use, tetracyclines have been extensively adopted as growth promoters and therapeutical drugs in animal husbandry, i.e., livestock, poultry breeding, and pigs' production (Kühne et al., 2000; Prado et al., 2009b; Wang et al., 2018). Multiple previous studies (Halling-Sorensen, 2001; Loke et al., 2002; Sengelov et al., 2003) illustrated the significant concentrations of tetracycline encountered in pig slurry (up to 5 mg/L). Even though the concern of the increasing microbial resistances to tetracyclines has been raised and the use of tetracyclines as growth promoters has been outlawed, statistics show that tetracyclines comprise more than 65% of the antibiotics prescribed for the therapeutic use of animals within the European Community (FEDESA, 1998). Also, oxytetracycline which falls under the tetracycline class has been reported as one of the most exploited antibiotics in aquaculture for the treatment of fish bacterial diseases such as furunculosis, aeromonosis, pseudomonosis, lactococcosis, and vibriosis (Ali Abadi & MacNeil, 2002; Cenavisa, 2016; Drugs.com, 2016; Leal et al., 2019).

As for human therapy, tetracyclines can be employed to treat several infections, such as respiratory infections like atypical pneumonias, community-acquired pneumonia, rickettsial and chlamydial infections, Lyme disease, cholera, syphilis, periodontal infections, uncomplicated genital Chlamydia trachomatis infections, acute Q fever, penicillin-resistant Streptococcus pneumonia, as well as the treatment of acne particularly acne vulgaris (Ochsendorf, 2010; Saikali & Singh, 2003; Smilack, 1999). They have also been used for the treatment of malaria due to Plasmodium falciparum (Eliopoulos et al., 2003). In addition, Smith & Rajan (2000) suggested that tetracyclines could be effective for treating people infected with filarial nematodes, while Chopra and Roberts (2001) showed

that tetracyclines antibiotics are beneficial to treat infections with Entamoeba histolytica, Giardia lamblia, Leishmania major, Trichomonas vaginalis, and Toxoplasma gondii.

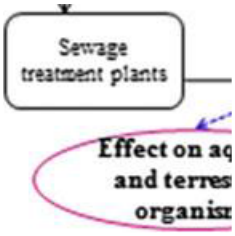
Speaking about the agricultural aspect, tetracyclines get delivered into the soil through land application of farmyard manure as plant nutrient sources (Wu et al., 2013). The presence of tetracycline (TC), a compound within the tetracyclines family, in feces and urine is relatively significant as TC is poorly adsorbed in the digestive tract of animals resulting in the excretion of 50–80% in feces and urine (Tolls, 2001). In human medicine, tetracyclines are customarily prescribed between 300 and 1,000 mg/day (de Sousa, 2005). After TC ingestion, a substantial amount is excreted as active metabolites ending up in municipal wastewater systems (Seifrtová et al., 2009). According to Daghrir & Drogui (2013), after ingestion, more than 70 % of tetracyclines are egested and released unmetabolized into the environment through urine and feces from humans. Regarding hospital use, Pena et al. (2010) demonstrated based on analyzing four hospital wastewater effluent samples in Coimbra, Portugal that hospital effluent has an important contribution to the pharmaceutical load in the influent of the WWTPs. Pena et al. (2010) epitomized, from multiple previous studies, the tetracyclines concentrations in surface waters and wastewaters from WWTPs in some countries (Figure 3). Whether tetracyclines are directly released to surface waters, added to soil and passed on to groundwater, or discharged into the sewage system then WWTP to be discharged afterwards to rivers and streams, somehow or other they find their way to the environment. Figure 4 presented in a review by Daghrir & Drogui (2013) gives a rundown on the potential sources and pathways for the occurrence of tetracyclines in the environment.

Figure 3 – Levels of TCs in Environment Wastewaters in Different Countries (Pena et al., 2010)

Country	Matrix	Antibiotic	Levels ($\mu g l^{-1}$)
Sweden	WWTPs wastewater	Doxycycline	0.064–2.480 [a]
Luxembourg	WWTPs wastewater	TC	1.0–85.0 [b]
		OTC	1.0–7.0
	Rivers	TC	1.0–8.0
		OTC	1.0–7.0
Canada	WWTPs wastewater	TC	0.151–0.977 [c]
		DC	0.038–0.046
USA			
	Surface water	CTC	0.42–0.69 [d]
		TC	0.11
		OTC	0.34
	WWTPs wastewater	TCs	0.05–1.14 [e]
	WWTPs wastewater	TC	0.62 [f]
(Western NY)	WWTPs wastewater	TC	0.14–0.56 [g]
(Wisconsin)	WWTPs wastewater	TC	0.05–1.2 [h]
(Colorado)[i]	WWTPs wastewater		
	Influent	TC, CTC, DC, DMC	0.05–0.27
	Effluent	CTC	0.06
		DC	0.07

[a] Lindberg et al. (2004)
[b] Pailler et al. (2009)
[c] Miao et al. (2004)
[d] Kolpin et al. (2002)
[e] Yang & Carlson (2004)
[f] Batt & Aga (2005)
[g] Batt et al. (2006b)
[h] Karthikeyan & Meyer (2006)
[i] Yang et al. (2005)

Figure 4 – Potential Sources and Pathways for the Occurrence of Tetracyclines in the Environment (Daghrir & Drogui, 2013)

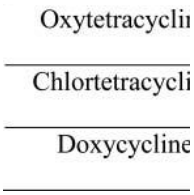


2.5.2 Structures and Characteristics of Tetracyclines

Tetracycline antibiotics, in general, have manifested efficiency against a huge collection of different microorganisms such as gram-positive and gram-negative bacteria, spirochetes, obligate intracellular bacteria such as rickettsiae and chlamydiae, mycoplasmas, and protozoan parasites (Chopra & Roberts, 2001). They are made up of four linearly annelated six-membered rings, namely 1,4.4a,5,5a,6,11,12a-octahydronaphthacene containing a characteristic arrangement of double bonds (Dürckheimer, 1975). The difference between the commonly used types of tetracyclines is only at the level of functional groups at three positions on the antibiotic backbone (Behal & Hunter, 1995). According to Boothe (2016), there are three naturally occurring tetracyclines (oxytetracycline, chlortetracycline, and demethylchlortetracycline) and several that are derived semi-synthetically (tetracycline, rolitetracycline, methacycline, minocycline,

doxycycline, lymecycline, etc). In addition, tetracycline-related antimicrobials called glycylcyclines have been lately introduced; these are represented by: 1) tigecycline which carries a bulky side chain compared with minocycline and 2) two new tetracycline antibiotics, omadacycline and eravacycline, which have been enlisted under clinical development in recent years (Grossman, 2016). However, Grossman (2016) classified tetracycline as one of the natural products produced by streptomycetes. Even though different studies presented and discussed various members of the tetracycline family (Behal & Hunter, 1995; Dürckheimer, 1975; Grossman, 2016; Nelson & Levy, 2001), some of them obsolete and others lately or currently being established, chlortetracycline, oxytetracycline and tetracycline were the most frequently used worldwide (López-Peñalver, 2010; Jeong et al., 2010; Halling-Sorensen et al., 2002). Jeong et al. (2010) also enlisted doxycycline among the most common TCs. Figure 5 shows the structural formulas of the 4 popular tetracyclines in use (Wang et al., 2008).

Figure 5 – Chemical Structures of Common Tetracyclines (Wang et al., 2008)



The popularity of tetracyclines in diverse sectors could be attributed, besides being a broad-spectrum antibiotic and not having major adverse side effects (Chopra & Roberts, 2001), to them being one of the cheapest classes of antibiotics available nowadays, driving them to be engaging for use in developing countries with small health care budgets (Eliopoulos et al., 2003). Thus, tetracyclines are classified as the second most common antibiotic worldwide and the first in China in terms of production and consumption owing to their low cost and easy synthesis (Daghrir & Drogui, 2013; Gu & Karthikeyan, 2005; Hao et al., 2012). They are yellow, odorless, bitter, light-sensitive, crystalline compounds of amphoteric nature because of their acidic components and the basic dimethylamino group (Dürckheimer, 1975; Mirzaei et al., 2013). Tetracycline antibiotics are highly hydrophilic (octanol/water partition coefficient log K_{OW} of -0.62, -1.12, -1.25 and -0.02 chlortetracycline, oxytetracycline, tetracycline, and doxycycline respectively) and have low volatility (Henry's law constant of in the range of 4.66×10^{-24} and 3.91×10^{-26} atm m^3/mol for the commonly used antibiotics), resulting in their persistence in the aquatic environment (Daghrir & Drogui, 2013; Sierra et al., 2021).

2.6 Tetracyclines Removal from Municipal Wastewater

2.6.1 Different Treatment Techniques for Tetracycline Removal from Municipal Wastewater

Various studies have examined the removal of tetracycline antibiotics from municipal wastewater using diverse treatment systems and different operating conditions. Gao et al. (2012a) investigated the elimination of fifteen pharmaceuticals in a treatment facility in Michigan employing conventional biological treatment; among these were chlortetracycline

and tetracycline which demonstrated > 99% reduction, as well as doxycycline and oxytetracycline which demonstrated < 50% reduction. They also verified a significant contribution of biodegradation to their removal mechanism. Gao et al. (2012b) also determined the removal efficiency of four tetracyclines via a conventional activated sludge WWTP based in Michigan as well. The total concentration of tetracyclines in the raw influent was determined as 1129.2 ng/L which decreased to 652.6 ng/L in the final effluent post disinfection, translating into a 42.2% elimination. However, the operating conditions (SRT, HRT, etc.) of the treatment system were not specified which limits the conclusions that could be made in this regard as some studies clearly proved the dependency of tetracyclines removal in WWTPs on the operating conditions with SRT being a more significant consideration (Kim et al., 2005). Kim et al. (2005) also substantiated sorption being the principal removal mechanism of tetracycline in activated sludge, disregarding photodegradation (which is one of the main transformation reactions of tetracyclines in the environment), isomerization, and epimerization (the latter two being reversible and highly pH dependent abiotic transformations of tetracyclines). Even though tetracyclines have high water solubility and low log K_{OW} coefficients, their fast partitioning onto suspended matter could be considerably attributed to ionic interactions and the metal-complexing properties of tetracyclines as is the case for TCs in soils (Tolls, 2001), in addition to hydrophobic interactions which still take part in sorption of soils at certain pH values (Kulshrestha et al., 2004). Lee et al. (2003) and Liao et al. (2001) further noticed that biomass hydrophobicity increases at higher SRTs.

Norvill et al. (2017) studied the degradation of tetracycline, administered into domestic wastewater at a concentration of 100 μg/L, in an outdoor pilot-scale high-rate algal pond (HRAP). At an average biomass concentration of 1.2 ± 0.1 g_{TSS}/L and 80 ± 4% chemical oxygen demand removal, tetracycline removal surpassed 93% at an HRT of 6 days

and 99% at an HRT of 7 days predominantly by the action of photodegradation. Even though sorption prevailed at night in terms of tetracycline elimination, it still contributed to less than 6% of the total removal. As for biodegradation, it was relatively negligible in contrast to what Gao et al. (2012a) conveyed.

Topal et al. (2016) investigated the removal efficiencies of TC and degradation products (4-epitetracycline (ETC), 4-epianhydrotetracycline (EATC), anhydrotetracycline (ATC)) and the physicochemical parameters affecting them in a municipal WWTP located in Elazığ, Turkey. The results showed highly variable removal efficiencies at different points in time falling in the range 11.21−79.65% except for ETC which showed no removal at all. This variation was due to instable operation of the WWTP and variability in the treated wastewater composition. Multiple studies have also shown small TC removal (zero to < 30%) (Lindberg et al., 2005; Batt et al., 2006a; Gulkowska et al., 2008; Watkinson et al., 2009). Nonetheless, other studies observed significant TC removal; for instance, Karthikeyan & Meyer (2006) specified a 67.9%–100% reduction in the concentration of dissolved tetracycline after secondary wastewater treatment (activated sludge, oxidation ditch, aerated lagoons and seepage cells) in multiple wastewater treatment facilities in Wisconsin.

Speaking about the efficiency of advanced technologies in TC removal, Gopal et al. (2020) presented a review on the efficiency of various advanced treatment techniques that have been employed for TC removal (Figure 6). Experiments have shown than different AOPs (photolysis, heterogeneous photocatalysis, fenton/photo-Fenton mediated processes, persulphate and peroxymono/disulphate mediated processes, photocatalysis with H_2 evolution, ozonation, and simultaneous TC degradation and adsorption) could be very effective for TC removal such that the removal efficiency could exceed 90% for certain schemes. Adsorption processes also manifested high applicability and involved a variety of

adsorbents with varying yet high and sometimes complete TC removal. On the other hand, coagulation and flocculation have been reported to exhibit less than 20% removal capacity. As for membrane filtration methods, even though they present potency in antibiotic removal, their fouling potential and high operational costs and energy input involved bound their adoption which is also the case for reverse osmosis.

Figure 6 – Advanced Treatment Techniques for TC Removal (Gopal et al., 2020)

The huge variability of the removal of tetracyclines in conventional biological wastewater treatment technologies affirms the intricacy of the elimination process in terms of the numerous direct and indirect factors, parameters, and structural elements contributing to the removal mechanism, whether related to the compound composition and arrangement or the configuration of the treatment system.

2.6.2 Aerobic MBRs Use for Tetracyclines Removal from Municipal Wastewater

The removal and fate of various emerging micropollutants, one of which being tetracycline, by MBR systems has rarely been studied in the past which necessitates its investigation in detail (Gurung et al., 2019). Gurung et al. (2019) examined the removal of

23 emerging contaminants, including tetracycline, identified in municipal wastewater by a pilot-scale membrane bioreactor (MBR) run at two different SRTs of 60 and 21 days while maintaining nearly constant permeation flow rate and transmembrane pressure (TMP) (4 – 5 kPa). The results affirmed the general conclusion drawn by previous researchers on SRT being a very influential operational parameter in MBR processes in terms of OMP removal (Clara et al., 2005; Schröder et al., 2012; Taheran et al., 2016; Tambosi et al., 2010); however, for tetracycline in particular, a consistently high removal of 98% was attained for both SRTs which is analogous with the results obtained by Kim et al. (2014). Generally, literature reports four main pathways resulting

in the elimination of OMPs by MBR processes; these are: (i) biotransformation or degradation (photodegradation or biodegradation); (ii) sorption onto the sludge; (iii) volatilization or stripping by aeration; and (iv) physical retention by membranes (Cirja et al., 2008; Li et al., 2015). Kim et al. (2014) looked into the first two pathways and identified that sorption to sludge is responsible for the entire 97% evaluated tetracycline removal with zero contribution to degradation/transformation which is in opposite on to what Gurung et al. (2019) reported about tetracycline having a high degree of elimination via biotransformation. As for volatilization, Gurung et al. (2019) declared that since Henry's law constant (K_H) values for tetracyclines are low ($<10^{-6}$), their reduction by volatilization is negligible. Concerning membrane retention, the typical molecular weight cut-off (MWCO) of MF and UF membranes are substantially higher (by several thousand daltons (Da)) (Taheran et al., 2016), so the physical retention of OMPs (MWCO between 200 and 800 Da) like tetracyclines in the MBR process is not probable.

Even though multiple studies affirmed that sludge adsorption is the main removal mechanism of tetracycline in conventional WWTPs such as Batt et al. (2007) who investigated the occurrence of tetracycline (TC) in four full-scale WWTPs and Li & Zhang

(2010) who examined tetracycline elimination in two activated sludge systems treating freshwater and saline sewage, former experimentations validated that bacterial communities in MBRs were notably different from those in the CAS systems (Luxmy et al., 2000; Baek & Pagilla, 2009). This could bring about variable results and altered conclusions when it comes to the contribution of each of the presented removal mechanisms.

The overall preferability of MBRs over CAS processes in terms of antibiotics removal has been affirmed and attributed to their higher biomass and presence of specialized microorganisms that better reduce micropollutants (Meng et al., 2012). For tetracycline precisely, Tran et al. (2016) evaluated TC elimination by an MBR to be 83.3~95.5% which is significantly higher than what was reported for CAS systems (44.3~87.6%). Xu et al. (2017) deduced that the MBR system could reduce more than 90% of TC found in wastewater at a range of 1 to 1000 μg/L without having a notable effect on nutrient removal if present at environmentally relevant concentrations; however, TC occurrence decreases diversity of the microbial community and manifests an inhibitory effect on bacterial species. Even though the influent wastewater was synthetic, it had a COD of 435.2 ± 92.2 mg/L which is representative of municipal WW. The removal route was bio-adsorption in correspondence with earlier research; as for hydrolysis, which is another abiotic removal mechanism besides adsorption, it could be ignored because TC is a stable compound (Xu et al., 2017). This high adsorption potential of TC to activated sludge could be contributing to the persistent toxicity of TC to microorganisms.

2.6.3 Anaerobic MBRs Use for Tetracyclines Removal from Municipal Wastewater

Although multiple experiments looked into the removal of tetracycline from municipal wastewater influent using an aerobic MBR, the anaerobic configuration of the

MBR (AnMBR) in this scheme hasn't been exclusively inspected. Even though the aim of the research conducted by Fakhri et al. (2021) was to study the impact of Trichocladium canadense, a saprotrophic genus of fungi belonging to the family Chaetomiaceae, as a bioaugmentor on the microbial community structure and the performance of an AnMBR treating synthetic pharmaceutical wastewater, the control symbolized a typical AnMBR being fed antibiotic-containing influent constituting of erythromycin, sulfamethoxazole, and tetracycline. TC removal was appraised at 96.13% with a substantial contribution of adsorption to biofilm. However, these results are not enough to draw out conclusions on the sole elimination of TC by an AnMBR system. This is due to several reasons, one of which is the fact that the combination of antibiotics is probably one of the influencing elements in the elimination and accumulation of antibiotics. Also, the influent was provided with 37.3 mgTC/L which is way more than its environmentally relevant concentration. The removal efficiency of antibiotics not only depends on the operational conditions of the treatment system and the hydrophobicity and molecular structure of the antibiotic, but it is also influenced by the initial concentration and combination of antibiotics, as per Tran et al. (2018). This is besides the role that the type and chemical as well as the biological composition of the wastewater play leading to a probable dissimilarity in TC removal between treating municipal wastewater containing antibiotics and synthetic pharmaceutical wastewater.

Other studies looked into tetracycline removal in an anaerobic digester, aerobic MBR, or hybrid anoxic/aerobic MBR system, or from different types of wastewater other than municipal wastewater, leaving the solitary focus on tetracycline removal in a classic AnMBR out. Varel et al. (2012) specified chlortetracycline removal efficiencies of 7%, 80%, and 98% in anaerobic digesters at 22°C, 38°C, and 55°C, respectively. Xiong et al. (2017) evaluated the percentage of elimination of tetracycline hydrochloric acid added in

three different concentrations into synthetic wastewater fed to separate anaerobic batch systems. For all three concentrations of 1 μg/L, 150 μg/L, and 20 mg/L reflecting TC presence in domestic wastewater (Yang et al. 2005), hospital wastewater (Pena et al. 2010), and livestock wastewater (Álvarez et al. 2010), respectively, total tetracycline hydrochloric acid was substantially removed with an overall efficiency of 85 ± 2%. Hybrid systems involving TC include research performed by Zhu et al. (2018) and Zhu et al. (2017); as for experiments involving different types of wastewaters, Kaewmanee et al. (2019), Prado et al. (2009b) and others can be pointed out. In short, the examination of an AnMBR performance being fed municipal wastewater containing tetracycline and the study of the fate of this antibiotic still forms a gap that needs to be filled in the field of research taking into account the plentiful economical and performant advantages that adopting AnMBRs as treatment systems has.

2.6.4 The Challenges of Tetracycline Removal from Wastewater

Environmental scientists, engineers, and researchers have given special attention to studying tetracycline removal from wastewater as this phenomenon poses some challenges due to the nature and state of presence of this antibiotic in aqueous environments. TCs possessing biological toxicity are omnipresent in wastewater and occur in the aquatic environment persistently due to the fact of their being incompletely metabolized in humans and animals, difficult to degrade, highly hydrophilic, and manifesting low volatility (Daghrir & Drogui, 2013; Michael et al., 2013; Watkinson et al., 2007). Liao et al. (2021) reviewed the impact of TC on the active bacteria present in the sludge matrix and the proliferation of tetracycline-resistance genes. Katipoglu-Yazan et al. (2013) showed that high concentration of antibiotics in urban wastewater yields a substantial inhibitory effect

on microorganisms in sewage treatment systems, which was emphasized for TC particularly by Grabert et al. (2018) who reported that TC can selectively hinder or influence the functional expression of bacteria during wastewater treatment. The adverse ramifications of TC on activated sludge in different treatment systems were studied, each of which might or might not apply to anaerobic sludge of AnMBRs. For instance, Liao et al. (2001) pointed out that the sludge activity can be fundamentally governed by activated sludge settling properties, particle size and odor which are altered by the prompt stringent effects of TC. Katipoglu-Yazan et al. (2015) found that TCs express biological toxicity, which could be irreversible, in the aqueous environment by eradicating the integrity of sludge which causes floc disintegration and cellular rupture. However, in case of sustained exposure to TC, the sludge might be triggered to protect active bacteria by mass increases from severe exposure to exogenous emerging micropollutants (Song et al., 2016; Wang et al., 2018) and coagulation into more compact flocs (Yang et al., 2016). Therefore, extensive concerns are brought up when tetracycline occurrence and removal from wastewater is addressed.

2.7 Tetracycline and Antibiotic Resistance

2.7.1 Impact of Tetracycline on Antibiotic Resistance in WWTPs

Because antibiotics are generally partially metabolized by humans and animals' bodies with a 30% estimated metabolic rate (Kümmerer & Henninger, 2003), abundant amounts of antibiotics and their metabolites are potentially released via diverse routes into the environment, thus tremendously contributing to the evolution, spread, and selection of antibiotic resistance in bacterial pathogens (Allen et al., 2010). In general, antibiotics are emerging micropollutants that can select antibiotic resistance at lethal or non-lethal

concentrations (Andersson and Hughes 2012; Lupo et al., 2012). Zhang et al. (2019) evaluated the effect of tetracycline added into a sequencing batch reactor (SBR) at multiple concentrations within the range 0-500 μg/L on microbial communities and ARG proliferation in aerobic granular sludge. The results showed efficient tetracycline removal (between 84.8 ± 6.8% and 94.2 ± 2.7%), nonetheless, it was found that TC, even when occurring in minimal concentrations of μg/L, could substantially augment the absolute and relative abundances of *tetA*, *sulII*, and *blaTEM-1* in the effluent and aerobic granules. This ratiocinates the influence of tetracycline serving as a selection pressure on the proliferation of ARGs corresponding to multiple types of antibiotics in aerobic granules. Sarmah et al. (2006) and Baquero et al. (2008) asserted that the potential contribution of tetracycline in escalating bacterial resistance has made the presence and release of TC antibiotics into the aquatic environment garner lots of attention. Nevertheless, a review by Daghrir & Drogui (2013) brought about the gap of availability of reliable studies that describe the relationship between the occurrence of environmentally relevant concentrations of TCs and antibiotic resistant microorganisms. Frequent and lengthy exposure of bacteria to sublethal dosages of antibiotics enhances this resistance (Kemper et al. 2008) after which the conveyance of these ARB is possible either by direct contact or through the food chain (Richter et al., 1996).

Because conventional WWTPs are not designed for removal of antibiotics generally, Auerbach et al. 2007 have pinpointed WWTPs as a fountainhead for the development, transmission, and spread of tetracycline ARGs in activated sludge. The results showed that the concentrations of *tetQ* resistance genes were highest in the wastewater influent, while *tetG* genes were highest in activated sludge.

2.7.2 Impact of Tetracycline on Antibiotic Resistance in AnMBRs

Even though few studies have appraised AnMBRs for their efficiency in the elimination of ARGs and ARB, only one study to date done by Zarei-Baygi et al. (2019) has looked into the effect and correlation of the occurrence and concentration of particular antibiotics with ARG proliferation or ARB abundances in AnMBR biomass and effluent, despite their addressed interrelationship in other wastewater treatment systems. The study scrutinized 3 antibiotics (sulfamethoxazole, erythromycin, and ampicillin) belonging to 3 different classes, but didn't include tetracycline. Nonetheless, conceptual conclusions drawn included the significant differences that were observed for ARG profiles between the biomass, biofilm, and the effluent regardless of the antibiotic addition phases in terms of nature and concentration which is similar to what other recent studies demonstrated (Zhang et al., 2018; Kappell et al., 2018; Munir et al., 2011; Wen et al., 2018; Zhu et al., 2018). Also, incremental antibiotic addition provoked an increase of most ARGs in biomass in contrast to ARB, the presence of which showed no notable sensitivity to antibiotic type and concentration in the effluent. Whether these conclusions can be generalized and applied for tetracycline or any other unstudied antibiotic addressed into an AnMBR system is something that requires exclusive examination.

Zhu et al. (2018) studied the combined effect, on five ARG subtypes (*sulI*, *sulII*, *tetC*, *tetX* and *ereA*) and *int1*, of tetracycline and sulfamethoxazole (SMX) (subsequent concentrations of 100 µg/L then 1000 µg/L each) added to synthetic wastewater fed into a a laboratory-scale anoxic-aerobic (A/O)-MBR, and the contribution of membrane foulants in this regard was addressed. Results showed that even though a vigorous ARG removal efficiency was attained with the abundance of reduced ARGs in the range of 0.6–5.6 orders of magnitude, the addition of SMX and TC enhanced ARG abundance by 0.5–1.4 orders of

magnitude in the AS and membrane fouling layer with ARGs in membrane foulants accounting for 13%–25% of the total absolute abundance of all analyzed samples in the MBR system under antibiotic exposure. Although it was notable that the presence of TC caused proliferation of the corresponding resistance determinants (*tet* genes) in the MBR system and consequently in the effluent, the copresence of TC and SMX and type of the MBR system (A/O) probably contributed to shaping these results. Therefore, sole investigation of tetracycline in an anaerobic setting of the MBR system still poses vagueness and imprecision that necessitates looking into.

CHAPTER THREE

AIM & OBJECTIVES

3.1 Research Aim

The aim of this research experiment was to evaluate the efficiency of a lab-scale AnMBR in removing tetracycline from municipal wastewater while yielding a high-quality effluent at the same time. The study also probed into the distribution of the eliminated tetracycline between different removal mechanisms (adsorption to sludge, biodegradation, adsorption to biofilm) through establishing a tetracycline mass balance before and up until full saturation of sludge with tetracycline. In addition, the effect of the presence of tetracycline on the proliferation of tetracycline-associated and other resistance genes in both their intracellular and extracellular forms of existence in the effluent was examined.

3.2 Research Objectives

The objectives of the presented study were:

1. Evaluate the overall performance of the AnMBR
   1.1. Assess the effluent quality (COD, VFAs, effluent methane)
   1.2. Observe the anticipated functioning of the system (VSS/TSS for sludge growth, methane production and progression of volatile fatty acids concentrations for effective anaerobic digestion, etc.) and the actual effect of tetracycline on its performance
2. Establish extraction, detection, and quantification methods for tetracycline from the effluent, sludge, and biofilm

3. Quantify tetracycline in the effluent & evaluate the overall removal efficiency of tetracycline by the AnMBR
4. Evaluate tetracycline adsorption

4.1. Quantify tetracycline in the sludge solids to evaluate adsorption to sludge

4.2. Quantify tetracycline in the membrane biofilms to evaluate adsorption to biofilm

5. Point out the role played by biofilms in tetracycline removal and compare different membrane types in this context
6. Construct a mass balance for tetracycline in the AnMBR system from which the percentage of biodegradation of tetracycline is deduced
7. Develop a suitable PCR procedure and quantify specific ARGs, both intracellular and extracellular, in the influent and effluent to yield visualization of changes in these ARG

3.3 Scope of Work

The study was performed using an AnMBR operated at 30℃. The AnMBR consisted of a 5 L reactor connected to 3 external membrane filtration units two of which were microfiltration units and one of which was an ultrafiltration unit. One of the MFs was an old membrane which has been operated for 47 days prior to the start of the experiment, while the second is a new unfouled membrane. The study was divided into two phases operated at approximately equal organic loading rate and extending over a course of 60 days. The first phase which spanned over 10 days involved feeding the reactor without tetracycline addition into the municipal wastewater influent, while the second phase introduced tetracycline into the influent at a constant concentration throughout the entire 50 days that followed. This

allowed, besides ensuring stable operation of the system, the identification of the effect tetracycline has on the performance and efficiency of the AnMBR and the variation in the ARG profiles between the two phases and the different membranes within the same phase. The removal of tetracycline was inspected more closely as the contribution of each of adsorption to sludge and biodegradation was assessed. The tendency of tetracycline to adsorb onto the biofilms, both loose and tight layers, developed on each of the two membrane unit sizes and each of the two MF membranes of different fouling layers was also examined.

CHAPTER FOUR

METHODOLOGY

4.1 Configuration and Operation of the AnMBR System

The AnMBR was composed of a continuously stirred tank reactor (CSTR), with a working volume varying between 3-3.6 L (Chemglass Life Science, USA) throughout the span of the experiment. Continuous mixing at 200 rpm was applied in the reactor using an internal impeller with curved blades. Mesophilic conditions were ensured at all times by keeping the reactor inside a water jacket connected to a water bath which maintains a water temperature of 30 °C.

Connected to the the reactor were three external crossflow membrane units: a microfiltration unit (MF1), nanofiltration unit (UF), and another microfiltration unit (MF2). The membranes were flat sheet polyvinylidene difluoride (PVDF) membranes of 0.2 µm pore size for the MFs and a molecular weight cut-off (MWCO) of 150 kDa for the UF membrane (Microdyn Nadir, Germany). They also had an effective area of 57 cm^2. During both phases, the effluent flow rate was approximately constant and equal for the two MF membranes (1.2 – 1.5 L/day) and slightly less for the UF membrane (1 – 1.4 L/day) translating into permeate fluxes of 8.9 - 11 L/m^2h for MF membranes and 7.5 – 10.5 L/m^2h for UF membrane. This variation allowed for analysis of the difference in the performance between different membrane pore sizes. On day 30 of the experiment, membranes UF and MF2 were harvested and new UF and MF2 membranes were set up.

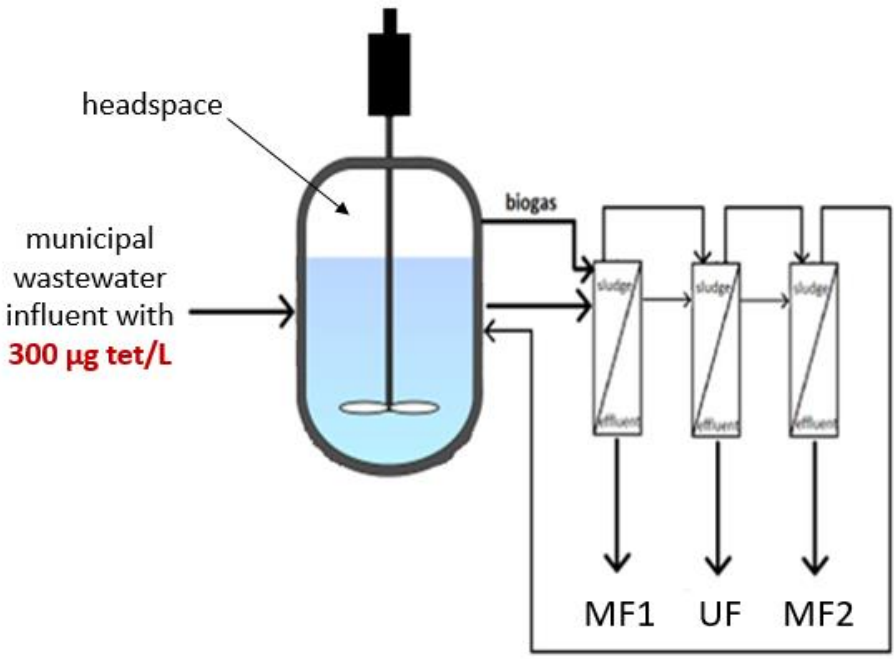
The sludge used for seeding the reactor was obtained from an anaerobic digestor in Lebanon, and its pH was maintained at 7. As for the sludge retention time (SRT), it was 490

days during the pre-tetracycline addition phase but was decreased to 148 - 223 days in the tetracycline addition phase during which substantial volume of sludge was withdrawn for tetracycline quantification purposes.

Besides relaxing the membrane for 60 seconds every 59 minutes and backwashing for 20 minutes daily to prevent membrane fouling, membrane scouring by continuous biogas sparging at 290 rpm across the membrane surface and continuous sludge recirculation at 690 rpm were implemented.

The reactor was fed with municipal wastewater with an influent chemical oxygen demand (COD) of 310 – 583 mg/L at an organic loading rate between 0.52 ± 0.08 g/L-d over the two phases. The wastewater was stored at 4°C prior to feeding into the reactor. As previously mentioned, no tetracycline was added into the influent during phase 1 (day 0 – 10), but during phase 2 (day 11 – 61), tetracycline was continuously added to the influent at a concentration of 300 µg/L.

Figure 7 – Set-up Configuration of the AnMBR System



4.2 Effluent Water Quality Testing

The water quality of the effluent was routinely assessed by testing for COD and volatile fatty acids (VFAs).

4.2.1 COD Testing for the Effluent

COD testing was done in accordance with the Reactor Digestion Method then measured by colorimetric determination using a Hach DR3900 Spectrophotometer. In short, 2 mL of the sample were pipetted into tubes containing dichromate and were heated in the COD digestor at 150 ℃ for two hours. The samples were allowed to cool to room temperature then measured at a wavelength of 600 nm. Prior to each spectrophotometer measurement, a blank, prepared similar to the aforementioned samples but with deionized water in place of the effluent, was used to zero the spectrophotometer serving as the calibration blank.

4.2.2 Quantification of VFAs in the Effluent

As for VFAs designating acetate, propionate, and butyrate, they were tested for once a week on a Metrosep Organic Acids - 250/7.8 (6.1005.200) column at flow 0.5 mL/min using an ion chromatograph (882 Compact IC Plus) with a conductivity detector along with an 858 Professional Sample Processor (Metrohm AG, Switzerland). Prior to VFA detection and quantification in the collected effluent samples, they were filtered using 0.2 µm Nylon syringe filters and sometimes diluted depending on the anticipated concentration range. Standards of respective concentrations of 5 mg/L, 25 mg/L, 50 mg/L, 100 mg/L, 250 mg/L, and 500 mg/L were prepared and run for VFAs to plot a standard curve, with R^2 value

greater than 0.95, for each VFA. A representative standard was run each time to calibrate the instrument and verify the consistency of its performance.

4.2.3 Solid Phase Extraction for Tetracycline Extraction from Effluents

Solid phase extraction was applied on the effluent in order to extract and concentrate tetracycline for better detection limit afterwards. A 100 mL sample of each of the three effluents was collected and filtered on a 0.45 μm membrane filter. The filtrate of each was then subjected to solid phase extraction at a pressure of -5 inHg to concentrate the analyte of interest, i.e. tetracycline. For that, HLB SPE cartridges of polyethylene material, having a bed weight of 200 mg and a particle size of 50-70 μm were used. First, the column was conditioned using 3 mL of methanol, then equilibration of the column was done using 2 mL of distilled water. Loading of the 100 mL sample followed at a rate of 3.5 mL/min, after which the residuals were washed away using 2 mL of distilled water. Finally, tetracycline was eluted by 3 mL of methanol at a rate of 5 mL/min. 1.5 mL of the eluted volume were transferred into an autosampler 1.5 mL vial to be run on HPLC-UV (adapted from Abbasi et al. (2012a)).

The correlation between peak areas corresponding to the same tetracycline concentration in each of the effluent sample and a 3 mL MeOH sample was determined, in addition to the recovery efficiency of SPE. Based on this, methanol standards spiked with tetracycline were used to generate a matrix-matched calibration curve.

4.2.3.1 Recovery Efficiency of SPE for Tetracycline Extraction from Effluents

The recovery efficiency of SPE was determined as follows:

- Two 100 mL effluent samples were collected from the effluent lines (before the phase of tetracycline addition).
- The samples were filtered on a 0.45 μm membrane filter.
- The filtrate of one of the samples was spiked with, for example, 60 µL of 0.5 g *tet*/L.
- The filtrate of each of the spiked and unspiked samples was subjected to solid phase extraction.
- After SPE, the final product of the unspiked sample was spiked with the same mass of tetracycline.
- The final product of both samples, spiked before SPE and spiked after SPE, were run on HPLC-UV; the ratio of the peak area yielded for the sample spiked after SPE to the peak area yielded for the sample spiked before SPE is the recovery efficiency of SPE extraction.
- This procedure was repeated 4 times using different concentrations of spiking to ensure consistency of results and that the recovery efficiency didn't vary with concentration of tetracycline present. The average of the 4 values was taken and adopted as the recovery efficiency of tetracycline during SPE for the effluent.

4.2.3.2 Relationship Between Used Standards and Standards in Effluent Matrix

The correlation between peak areas corresponding to the same tetracycline concentration in each of the effluent samples and a 3 mL MeOH sample was determined as follows:

- A 100 mL effluent sample was collected from the effluent lines (before the phase of tetracycline addition).
- The sample was filtered on a 0.45 μm membrane filter.

- The filtrate of the sample was subjected to solid phase extraction.
- The final product of the sample was spiked with, for example, 60 µL of 0.5 g *tet*/L.
- 3 mL of a mixture of MeOH was spiked with the same mass of tetracycline: 60 µL of 0.5 g/L.
- The two samples were run on HPLC-UV. The ratio of the peak area yielded for the effluent sample to the peak area yielded for the MeOH sample is the correlation between the the standards used and standards desired.
- This procedure was repeated 3 times to ensure consistency of results.
- Multiple MeOH standards spiked with different concentrations of tetracycline were prepared, and the peak areas of effluent samples spiked with the same concentrations were deduced.
- The peak area yielded for MeOH standards was multiplied by (1) recovery efficiency of SPE and (2) correlation factor with spiked effluent sample. The value yielded is thus plotted on the matrix-matched standard curve.

4.3 Sludge Testing

As previously mentioned, the pH of the sludge was regularly monitored and measured using a pH meter to maintain a value around 7.0. In addition, the total suspended solids (TSS) and volatile suspended solids (VSS) in the sludge were systematically measured following American Public Health Association (APHA) Standard Method 2540 described by Baird et al. (2017).

4.3.1 QuEChERS for Extraction of Tetracycline from Sludge Solids

A QuEChERS procedure, characterized and named based on being 'Quick, Easy, Cheap, Effective, Rugged and Safe', was applied on sludge in order to extract tetracycline. A 50 mL sample of sludge was withdrawn from the reactor then centrifuged at 14000 rpm for 15 minutes after which the supernatant was disregarded, and the pellet was air-dried for 20 to 25 minutes. 200 μL of distilled water were then added to the air-dried pellet, then vigorous mixing was applied using a vortex to ensure blending of the solids with water. Afterwards, the tube was placed in darkness overnight. The extraction procedure was done the next day during which the extracting solvents were added in succession: 10 mL of 0.2 M Na_2EDTA, 8 mL of ACN, and 2 mL of MeOH. The mixture was vortex-mixed for 15 seconds before 6.5 g of extraction salts were added consisting of 4 g $MgSO_4$, 1 g NaCl, 1 g Na_3Cit, and 0.5 g Na_2Cit, and the sample was shaken immediately. After vortex-mixing for 1 minute, the combination was ultrasonicated for 10 minutes at 30℃ then centrifuged at 3500 rpm for 10 minutes. From the supernatant organic phase, which is the top-most layer, 5 mL of volume was taken and added into a new tube containing 600 mg of dispersive SPE composed of 150 mg of primary secondary amine (PSA) sorbent and 450 mg of magnesium sulfate ($MgSO_4$). After shaking the mix manually followed by vortex-mixing for 1 minute, the sample was centrifuged at 1500 rpm for 5 minutes, and the supernatant was collected. The supernatant was then filtered with 0.22 µm PES syringe filter into a glass vial & evaporated to dryness under a gentle stream of nitrogen gas at 40°C. Finally, reconstitution was done with 2 mL of a mixture of ACN and distilled water (0.1% formic acid), 5/95, v/v. The final product was transferred into an autosampler 1.5 mL vial to be run on HPLC-UV (adapted from Ajibola et al. (2020)).

The correlation between peak areas corresponding to the same tetracycline concentration in each of the sludge sample and a 2 mL mix of ACN and distilled water (0.1% formic acid), 5/95, v/v was determined as well as the recovery efficiency of QuEChERS, and standards of ACN and distilled water (0.1% formic acid) mix spiked with tetracycline were used from which a matrix-matched curve was deduced.

4.3.1.1 Recovery Efficiency of QuEChERS for Tetracycline Extraction from Sludge Solids

The recovery efficiency of QuEChERS extraction for sludge was determined as follows:

- Two 50 mL sludge samples were withdrawn from the reactor (before the phase of tetracycline addition).
- The samples were centrifuged at 14000 rpm for 15 minutes.
- The supernatant was disregarded.
- The pellets were air-dried for 20-25 minutes.
- One of the two samples was spiked with, for example, 150 µL of 0.5 g/L, while the other was left unspiked.
- QuEChERS was done on both, spiked and unspiked samples.
- After reconstitution, the unspiked sample was spiked with the same mass of tetracycline, 150 µL of 0.5 g/L.
- The two samples were run on HPLC-UV; the ratio of the peak area yielded for the sample spiked after QuEChERS to the peak area yielded for the sample spiked before QuEChERS is the recovery efficiency of QuEChERS extraction for sludge.

- This procedure was repeated 4 times using different concentrations of spiking to ensure consistency of results and that the recovery efficiency didn't vary with concentration of tetracycline present. The average of the 4 values was taken and adopted as the recovery efficiency of tetracycline during QuEChERS extraction for sludge.

4.3.1.2 Relationship Between Used Standards and Standards in Sludge Matrix

The correlation between peak areas corresponding to the same tetracycline concentration in each of the sludge sample and a 2 mL mix of ACN and distilled water (0.1% formic acid), 5/95, v/v was determined as follows:

- A 50 mL sludge sample was withdrawn from the reactor (before the phase of tetracycline addition).
- The sample was centrifuged at 14000 rpm for 15 minutes.
- The supernatant was disregarded.
- The pellets were air-dried for 20-25 minutes.
- QuEChERS was done on the sample.
- After reconstitution, the sample was spiked with, for example, 150 µL of 0.5 g/L.
- 2 mL of a mixture of ACN and distilled water (0.1% formic acid), 5/95, v/v was spiked with the same mass of tetracycline: 150 µL of 0.5 g/L.
- The two samples were run on HPLC-UV. The ratio of the peak area yielded for the sludge sample to the peak area yielded for the ACN/DI water sample is the correlation between the the standards used and standards desired.
- This procedure was repeated twice to ensure consistency of results.

- Multiple ACN/DI water standards spiked with different concentrations of tetracycline were prepared, and the peak areas of sludge samples spiked with the same concentrations were deduced.
- The peak area yielded for ACN/DI water (0.1% FA) standards was multiplied by (1) recovery efficiency of QuEChERS for sludge and (2) correlation factor with spiked sludge sample. The value yielded was thus plotted on the matrix-matched standard curve.

4.4 Biofilm Testing

4.4.1 QuEChERS for Extraction of Tetracycline from Biofilm

A QuEChERS procedure, slightly varying from the one previously described for sludge, was applied on the biofilm in order to extract tetracycline from its loose and tight layers. A quarter of a membrane biofilm sample was put into a 15 mL tube. This applied to loose biofilm layer by scraping it off the membrane surface and to tight biofilm layer by cutting the membrane itself into small pieces. 200 µL of distilled water were then added to the air-dried pellet, then vigorous mixing was applied using a vortex to ensure blending of the solids with water. Afterwards, the tube was placed in darkness overnight. The extraction procedure was done the next day during which the extracting solvents were added in succession: 5 mL of 0.2 M Na_2EDTA, 4 mL of ACN, and 1 mL of MeOH. The mixture was vortex-mixed for 15 seconds before 3.25 g of extraction salts were added consisting of 2 g $MgSO_4$, 0.5 g NaCl, 0.5 g Na_3Cit, and 0.25 g Na_2Cit, and the sample was shaken immediately. After vortex-mixing for 1 minute, the combination was ultrasonicated for 10

minutes at 30℃ then centrifuged at 3500 rpm for 10 minutes. From the supernatant organic phase which is the top-most layer, 3 mL of volume was taken and added into a new tube containing 300 mg of dispersive SPE composed of 75 mg of primary secondary amine (PSA) sorbent and 225 mg of magnesium sulfate (M_gSO_4). After shaking the mix manually followed by vortex-mixing for 1 minute, the sample was centrifuged at 1500 rpm for 5 minutes, and the supernatant was collected. The supernatant was then filtered with 0.22 µm PES syringe filter into a glass vial & evaporated to dryness under a gentle stream of nitrogen gas at 40°C. Finally, reconstitution was done with 2 mL of a mixture of ACN and distilled water (0.1% formic acid), 5/95, v/v. The final product was transferred into an autosampler 1.5 mL vial to be run on HPLC-UV (adapted from Ajibola et al. (2020)).

4.4.1.1 Recovery Efficiency of QuEChERS for Tetracycline Extraction from Biofilms

The recovery efficiency of QuEChERS extraction for each of the biofilm loose and tight layers was determined as follows:

- Two biofilm samples (both loose or both tight) were put in 15 mL tubes (before the phase of tetracycline addition).
- One of the two samples was spiked with, for example, 100 µL of 1 g/L, while the other was left unspiked.
- 200 µL of DI water were added followed by vortex-mixing. QuEChERS was done on both, spiked and unspiked samples.
- After reconstitution, the unspiked sample was spiked with the same mass of tetracycline, 100 µL of 1 g/L.

- The two samples were run on HPLC-UV; the ratio of the peak area yielded for the sample spiked after QuEChERS to the peak area yielded for the sample spiked before QuEChERS is the recovery efficiency of QuEChERS extraction for biofilm.
- This procedure was repeated twice to ensure consistency of results. The average of the 2 values was taken and adopted as the recovery efficiency of tetracycline during QuEChERS extraction for biofilm.

4.4.1.2 Relationship Between Used Standards and Standards in Biofilm Matrix

The correlation between peak areas corresponding to the same tetracycline concentration in each of the biofilm sample and a 2 mL mix of ACN and distilled water (0.1% formic acid), 5/95, v/v is determined as follows:

- A biofilm sample is put in a15 mL tube (before the phase of tetracycline addition).
- 200 µL of DI water are added followed by vortex-mixing. QuEChERS is done on the sample.
- After reconstitution, the sample is spiked with, for example, 100 µL of 1 g *tet*/L.
- 2 mL of a mixture of ACN and distilled water (0.1% formic acid), 5/95, v/v is spiked with the same mass of tetracycline: 100 µL of 1 g/L.
- The two samples are run on HPLC-UV. The ratio of the peak area yielded for the biofilm sample to the peak area yielded for the ACN/DI water sample is the correlation between the the standards used and standards desired.
- This procedure is repeated twice to ensure consistency of results.

- Multiple ACN/DI water standards spiked with different concentrations of tetracycline are prepared, and the peak areas of biofilm samples spiked with the same concentrations are deduced.
- The peak area yielded for ACN/DI water (0.1% FA) standards was multiplied by (1) recovery efficiency of QuEChERS for biofilm and (2) correlation factor with spiked biofilm sample. The value yielded was thus plotted on the matrix-matched standard curve.

4.5 Chromatography for Detection & Quantification of Tetracycline in Effluent, Sludge, and Biofilm Samples

After tetracycline extraction, its detection and quantification in the prepared samples as well as the standards was done using high performance liquid chromatography with UV detection. The HPLC used was a WATERS HPLC, model 2690D, and the detector model was Waters 996 Photodiode Array Detector. The stationary phase constituted of a Thermo Scientific ODS Hypersil C18 250x4.6mm column having a particle size of 5 µm. As for the mobile phase, it was isocratic composed of three solvents: MeOH/ACN/0.01 M oxalic acid, 20/35/45, v/v/v. The flowrate of the eluent was set to 1 mL/min and the column temperature to 25°C. The system pressure was fluctuating around 1500 psi during the run. The sample run time was set to 20 minutes, a sample injection volume of 100 µL inputted, and tetracycline was eluted at a retention time of ≈ 10 minutes and was detected at a wavelength of 360 nm (adapted from Sokol & Matisova (1994)).

4.6 Headspace Biogas and Effluent Methane Testing

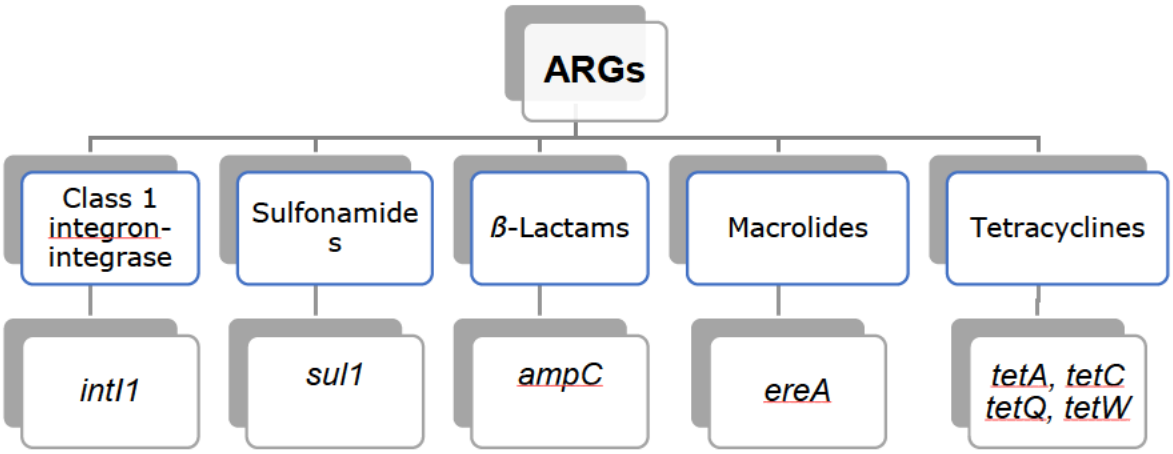
In order to quantify methane in the headspace and methane dissolved in the effluent, an Agilent 7890B gas chromatograph with thermal conductivity detection (GC-TCD) was used. The GC-TCD front detector temperature was set at 250 °C and the oven at 90 °C. For headspace biogas, a 300 mL biogas sample was withdrawn twice a week from the reactor headspace and stored in biogas bags. Then a 60 mL sample was taken from the biogas bag and injected into the GC-TCD.

As for methane dissolved in the effluent, the headspace technique was performed in order to obtain a representative sample to be injected into the GC-TCD. This was done by collecting a 32 mL effluent sample from the effluent line of each membrane directly into a syringe. The effluent sample was then introduced into an erlenmeyer flask previously sparged with nitrogen and having a gas bag connected to its rubber stopper via a needle. To mix the gaseous constituents, the flask was shaken and mixing was done using the syringe. The flask was heated for 10 minutes after which the flask was shaken again to allow dissolved methane into the gas phase so that excess methane volume would be admitted into the gas bag used for sampling.

4.7 Quantification of ARGs

To establish the intracellular and extracellular ARG profiles associated with each of the influent and effluent and deduce the effect of tetracycline presence on the proliferation of ARGs, quantitative PCR (qPCR) was employed. As tetracycline is the antibiotic under study, tetracycline associated ARGs were chiefly addressed in addition to other targeted ARGs conferring resistance to other classes of antibiotics (Figure 8). Those genes were targeted as they are usually abundantly found in wastewaters.

Figure 8 – ARGs Addressed and Quantified by qPCR in Influent & Effluent Samples



4.7.1 Effluent Sampling for ARG Quantification

For the effluent, permeate collection flasks were sterilized a night prior to sampling, and a representative sample greater than or equal to 500 mL was considered for each of the three effluents. After thoroughly mixing each of the three collected samples to ensure homogeneity, a volume of 120 mL of each permeate was taken and filtered on 0.45 µm membrane filters (Millipore, USA), then filters were stored at – 20°C for intracellular DNA extraction.

As for extracellular DNA extraction, the filtrate was spiked with 2 µL of pGEM 3-z vector (Promega, USA) then isopropanol was added on a 1:1 ratio after which the mix was incubated at – 20°C overnight. Following that, the mix was centrifuged at 14500 rpm for 30 minutes to pellet the eDNA; the supernatant was discarded and the pellets were washed with 70% ethanol. Finally, centrifugation was performed at the same speed of 14500 rpm for 20 minutes, the supernatant was discarded, and the pellets were air dried for 5 minutes then stored at – 20 °C until extraction (adapted from Zarei-Baygi et. al (2020)).

4.7.2 DNA Extraction, Quality Assessment, and ARG Quantification

DNA was extracted from the collected and stored samples (filters for iDNA and handled filtrate for eDNA) using DNeasy PowerSOil Kit (Qiagen, USA) according to the corresponding dictated procedure. After that, a Nanodrop ND 1000 spectrophotometer Version 3.3.0 was employed for evaluating the concentration and quality of the extracted DNA followed by PCR for ARG amplification.

For the PCR reaction, complementary primers and convenient thermocycling conditions for each gene were used (Table 1). A 20 µL PCR reaction volume composed of 4 µL of 5x FIREPol master mix (solis BioDyne, USA), 1 µL of each reverse and forward primer with a concentration of 5 µM, 2 µL of DNA template, and 12 µL of molecular grade water was adopted.

2 µL of the PCR product were used to run gel electrophoresis on a 1.5 % agarose gel, and the produced bands were visualized on a ChemiDoc Touching Imaging System (Bio-Rad Laboratories, USA) for the purpose of validating, based on base-pair length, that the targeted gene is the one that was amplified and thus ensure its presence.

Slightly modifying the corresponding standard procedure, the remaining 18 µL of the qPCR product were then purified using GenElute Gel Extraction Kit (Sigma-Aldrich, USA). An AccuGreen High Sensitivity dsDNA Quantitation Kit (Biotium) with a Qubit 2.0 Fluorometer (Thermo Fisher, USA) was then employed for the quantification of concentrations of purified genes. Thus, qPCR was performed on the purified product using the following mix: 10 µL Biotium Forget-Me-Not qPCR Master Mix, 1 µL of 5 µM primers, 1 µL of the template, and 7 µL of molecular grade water for a total mix of 20 µL after which the mix was run on CFX Connect Real-Time PCR Detection System (BioRad, USA). Calculation of the concentration of the purified genes is presented in Appendix A. To

demonstrate amplicon specificity, melting curve analysis was implemented by increasing temperatures from 65 °C to 95 °C at 0.5 °C intervals. It is important to note that samples were run in triplicate for verification. Amplification efficiency formulas can be found in appendix B. The thermocycling conditions used for qPCR were the same as those of PCR with the final elongation step. Lastly, the results were normalized to copies/mL as shown in Appendix B.

It should be pointed out that in order to prepare qPCR standards, municipal wastewater samples collected from different wastewater treatment plants in Lebanon were utilized to amplify the corresponding genes by qPCR.

Table 1 – Primers Used for PCR and qPCR with the Corresponding Thermocycling Conditions

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)
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)
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)

CHAPTER FIVE

RESULTS

5.1 Overall Reactor Performance Before & After Tetracycline Addition

The performance of the AnMBR was monitored regularly during both phases of the experiment for the purpose of: (1) comparing between the pre- and post-tetracycline addition phases for the sake of observing any change imposed by tetracycline exposure, and (2) ensuring good performance of the AnMBR prior to tetracycline addition to be able to draw out definitive conclusions on the efficiency of the system for tetracycline removal exclusively. Throughout the entire experiment, % COD removal, the variation of which is displayed in Figure 9, was 80 ± 8.27 %. During phase 1, a high COD removal of ≈ 90% was attained. After tetracycline started being fed to the reactor, % COD removal dropped to 71.58 %, but the system quickly started adapting and % COD removal started increasing again to reclaim its pre-tetracycline efficiency around 23 days after the start of phase 2. At day 47, a significant drop to 59.69% was recorded implying a disruption in the system which should have also been manifested in a decrease in the % of methane in the headspace. But unfortunately, due to technical problems in the GC-TCD during that period, no data on that is available (Figure 10). Figure 10 also shows that production of methane ranged between 0.334 L/day and 0.75 L/day throughout the experiment and the actual produced volume of methane was as expected except for two sampling points on days 29 and 32 which were likely due to sampling errors. Calculation of the expected methane production is shown in Appendix A. Regarding the VFA content, no VFA accumulation was recorded at any period of time; instead, null to negligible concentrations of < 15 mg/L were consistently reported for all three VFAs (acetate, butyrate and propionate). Speaking about TSS, its

concentration was 8.07 ± 0.35 g/L while a concentration of 6.66 ± 0.47 g/L was determined for VSS translating into a VSS/TSS ratio of 0.82 ± 0.02.

Figure 9 – Percentage of COD Removal Throughout the Experiment

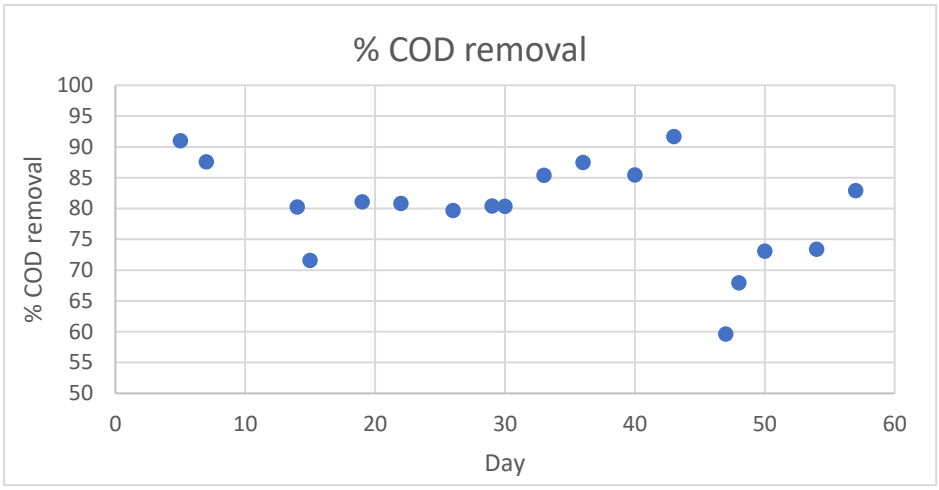
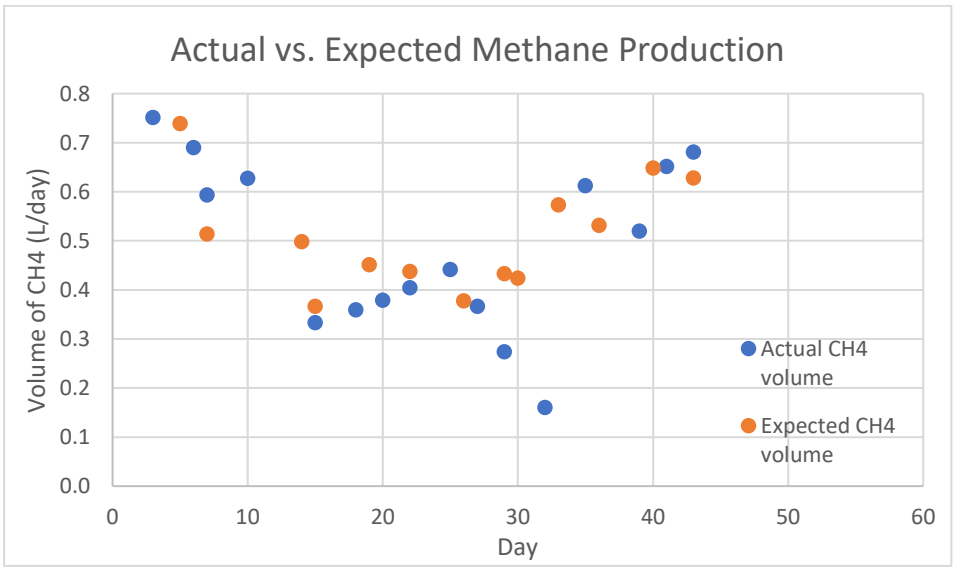


Figure 10 – Actual vs. Expected Volume of Methane Produced into the Headspace



The concentration concentration of methane dissolved in each of the three effluents was measured during the experiment (Figures 11, 12, and 13), and the results showed that after the start of phase 2 and exposure to tetracycline, an increase in effluent methane was determined for all three membranes but at different extents. MF1 permeate demonstrated a delayed above-

expected production of effluent methane; however, this increase was recorded for UF and MF2 permeates directly after the start of introduction of tetracycline. The gap between produced and expected effluent methane (Appendix A) increased in phase 2 which is probably attributed to a change in the microbial community triggered by tetracycline presence.

Figure 11– Actual vs. Expected Methane Dissolved in MF1 Effluent

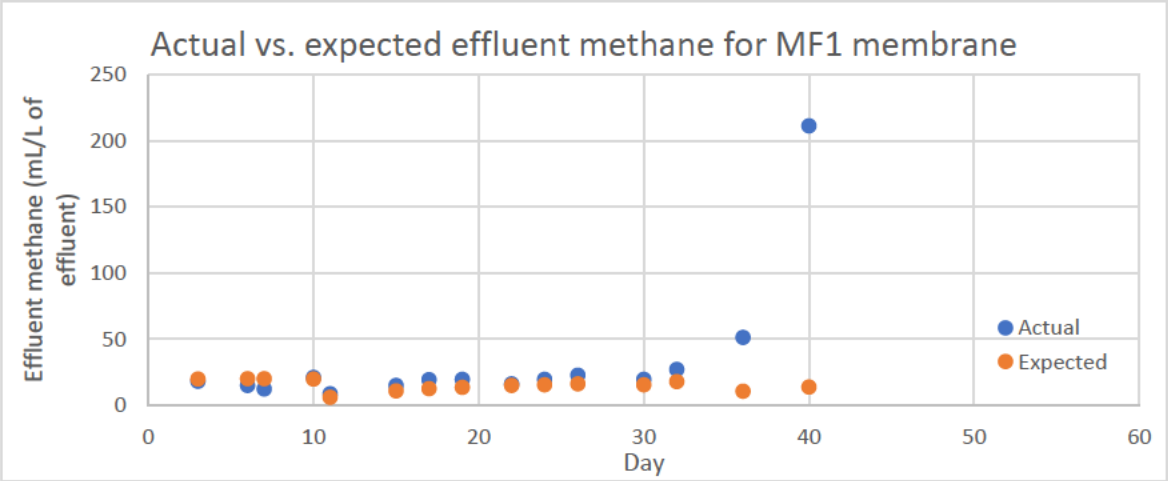


Figure 12 – Actual vs. Expected Methane Dissolved in UF Effluent

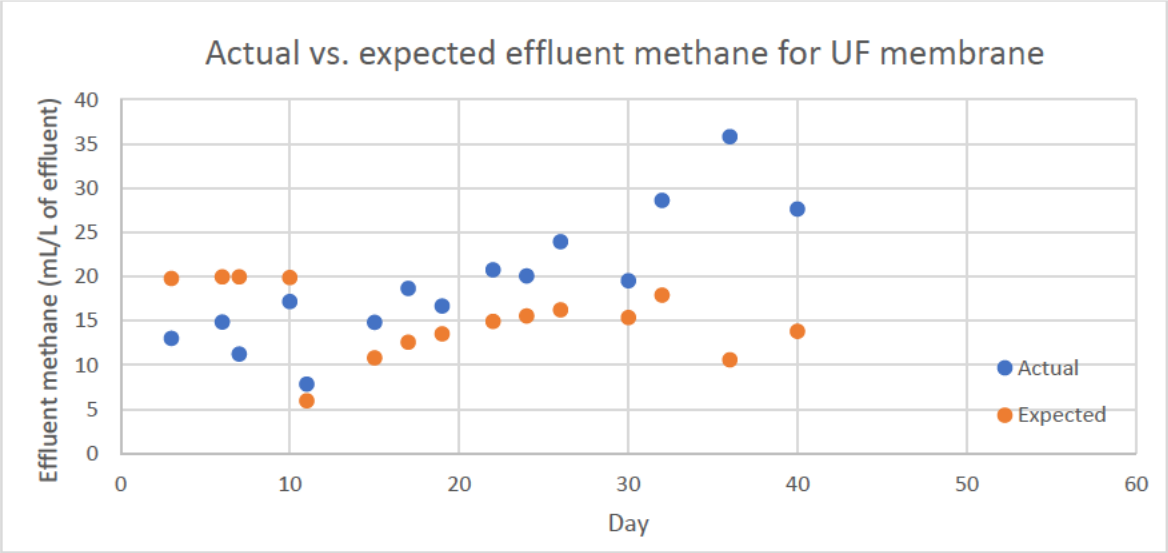
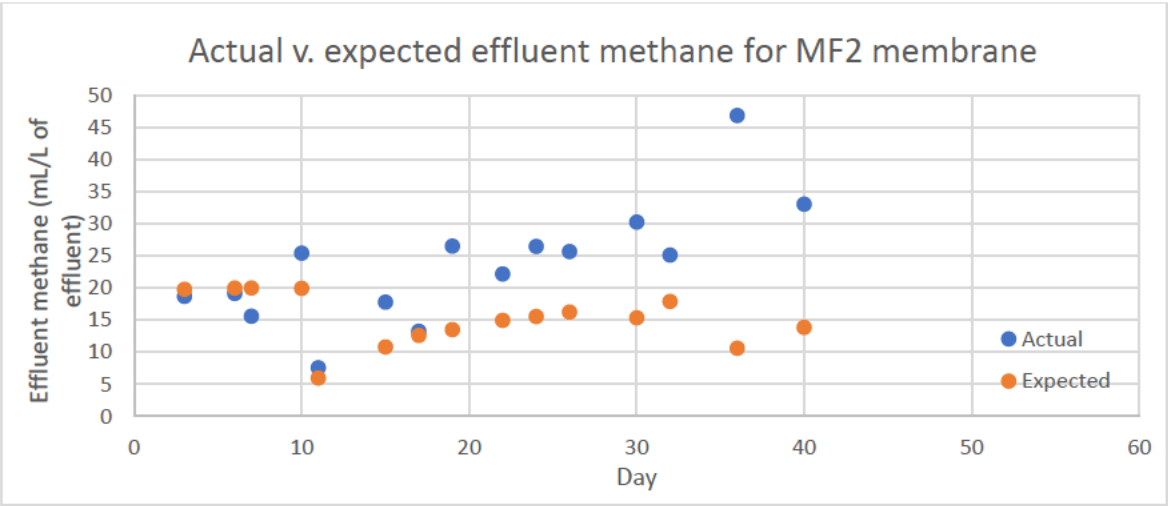


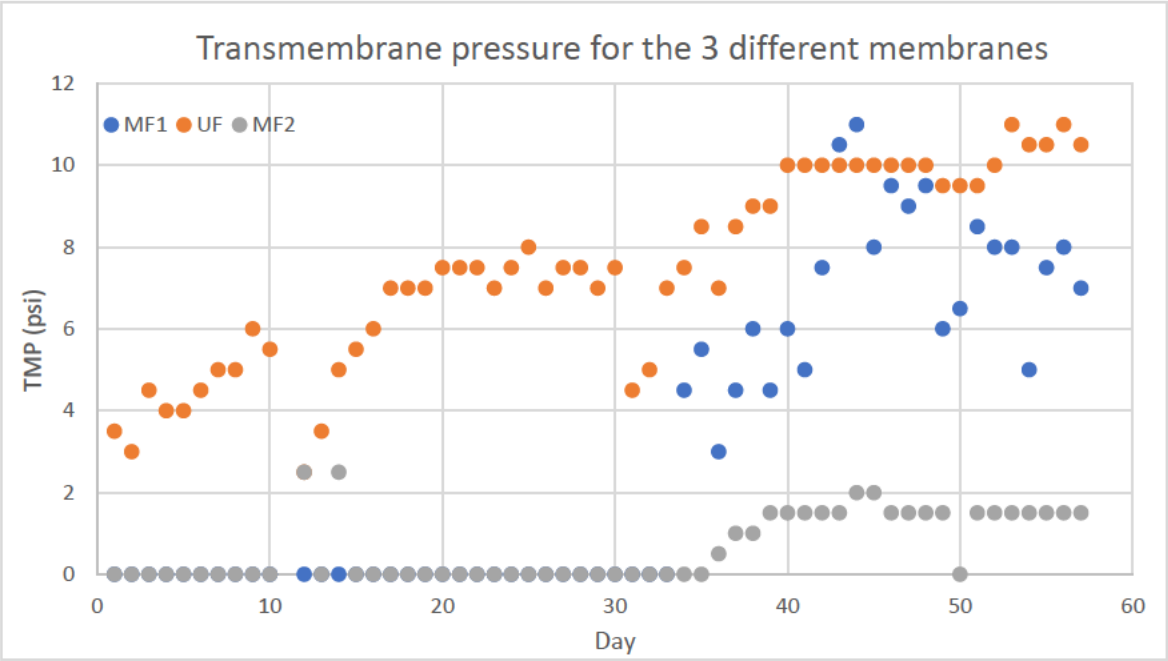
Figure 13 – Actual vs. Expected Methane Dissolved in MF2 Effluent



Speaking about the transmembrane pressure (Figure 14), the two MF membranes maintained a null TMP (except for MF2 on days 12 and 14 which is probably erroneous and explained by elevated baseline of the pressure gauge because of the null values recorded over the following 21 days) during phase 1 and the first 22-24 days of phase 2. The MF membranes then started building pressure, but MF1 which is older than MF2, recorded a higher TMP increase at all points than MF2. This is likely due to the fact that the longer operation of MF1 allowed the development of a thicker biofilm layer and is thus expected to foul earlier than

MF2. Regarding the UF membrane which has the smaller pore size, it started building pressure gradually from the beginning of the experiment, retaining a TMP higher than both MF membranes throughout the experiment. Ultimately, MF2 maintained a constant TMP of 1.5 inHg, while MF1 and UF manifested minor fluctuations in the TMPs around a value of 7-8 inHg for the former and 10-11 inHg for the latter.

Figure 14 – Development of Transmembrane Pressure for the 3 Membranes



5.2 Tetracycline Removal in the Three Effluents

Tetracycline was detected and quantified in the effluents of the three membranes for the purpose of evaluating the removal efficiency of tetracycline using the AnMBR system. Figure 15 displays the tetracycline concentrations in each of the effluents which is translated into Figure 16 representing the removal efficiency accomplished by each membrane. Overall, a high removal efficiency of a minimum of 89.95%, 77.39%, and 92.66% for MF1, UF, and MF2 respectively were attained throughout the tetracycline provision phase (Figure 16). The graphs

clearly show that the tetracycline concentration in the UF is persistently higher than both MF effluents, and this is likely ascribed to the nature of the microbial community that forms the biofilm of the UF and its distinctness from that developed on the surfaces of MF1 and MF2 membranes. Noting that UF has a smaller pore size than the MFs points out the lack of correspondence physical filtration to tetracycline removal and emphasizes what has been reported in literature about the improbability of physical retention of OMPs like tetracyclines in the MBR process (Taheran et al., 2016). The first detection of tetracycline was 12 days after phase 2 started for the three membrane effluents, signifying that between days 10 and 22, whatever tetracycline fed into the reactor was getting adsorbed to sludge and/or biodegraded by microorganisms. The general trend showed an increase in concentration of TC in the effluents with time. On day 46, a peak was observed at which concentrations of 30.14 µg/L, 67.82 µg/L, and 22.03 µg/L were evaluated for MF1, UF, and MF2 respectively. This was due to a decline in the performance of the AnMBR system manifested by a sudden drop in the % removal of COD (Figure 9) which could be by cause of various reasons such as a sudden leak or pervasion of oxygen disrupting the activity of microorganisms and curtailing anaerobic digestion. After day 50, the concentration of tetracycline in the effluents and thus the removal efficiency of tetracycline seemed to nearly stabilize settling at an average concentration of 17.6 µg/L, 32.23 µg/L, and 13.85 µg/L corresponding to an average removal efficiency of 94.13%, 89.26%, and 95.38% for MF1, UF, and MF2 respectively. The tetracycline concentration in the effluent of MF1 which is older was consistently a bit higher than that in the effluent of MF2, which negates the hypothesis of the thicker biofilm layer contributing to a better tetracycline removal. Also, the difference between the two concentrations at each sampling point is so minor that it could be within the margin of error of the tetracycline extraction and concentration procedure (SPE).

Figure 15 – Tetracycline Concentration in the Effluents of the Three Membranes During Phase 2

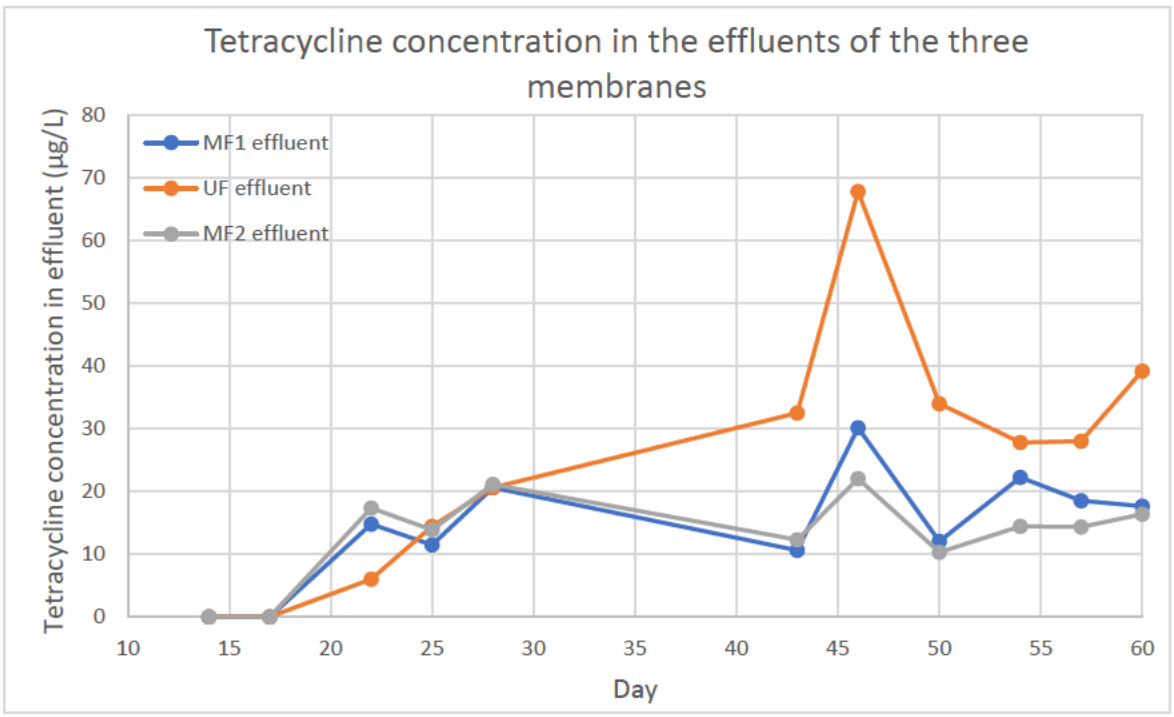
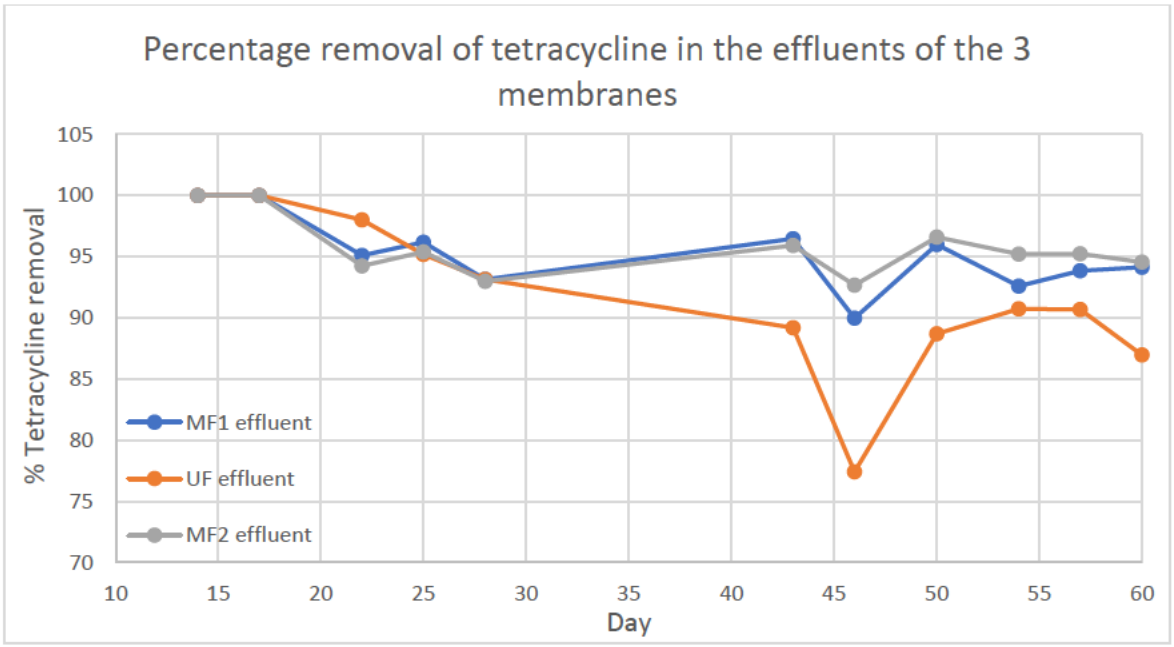


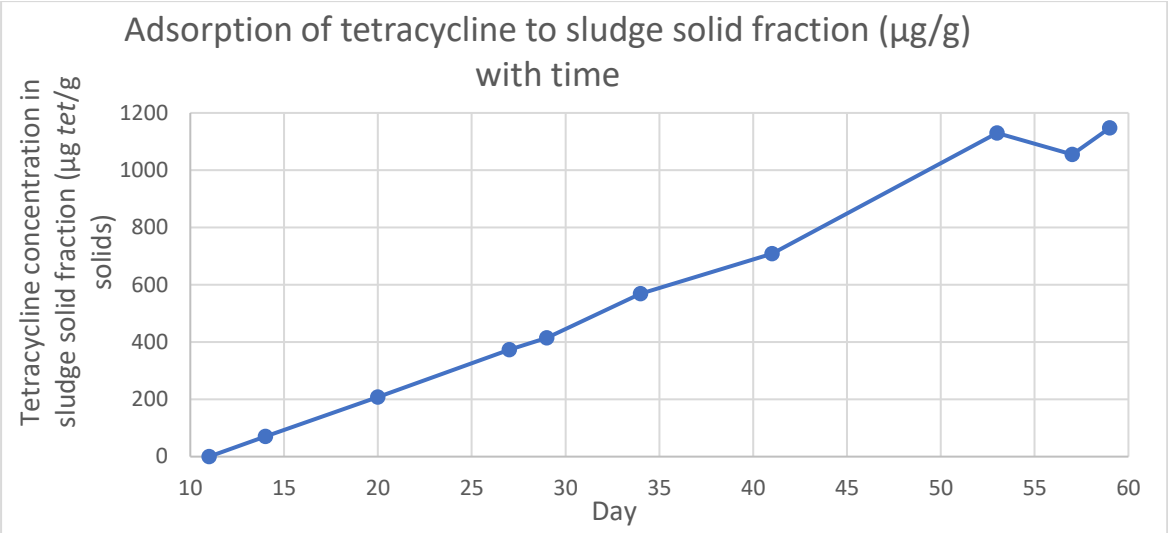
Figure 16 – Percentage Removal of Tetracycline in the Effluents of the Three Membranes During Phase 2



5.3 Evaluation of Tetracycline Adsorption to Sludge

The contribution of tetracycline adsorption to sludge to the removal of tetracycline from the wastewater influent was evaluated after running QuEChERS-extracted sludge samples on HPLC-UV (Figure 17). Chromatography results were yielded in terms of µg TC/L sludge, but assuming negligible presence of tetracycline in the sludge liquid fraction, which is in the range of that of the effluents, and taking into consideration the TSS content of the sludge, the graph (Figure 17) was constructed on a µg TC/g solids basis. The graph shows that directly after tetracycline started being fed into the system, tetracycline started adsorbing to sludge fast and registered a concentration of 70.2 µg TC/g solids within 4 days. The increase in the adsorption of tetracycline to sludge was linear throughout phase 2 until the sludge reached full saturation of 1129.7 µg TC/g solids on day 53 after which a plateau was illustrated. Beyond that, the tetracycline concentration in the effluents, if further testing was done past day 60, is expected to increase as no removal would further be attributed to adsorption and biodegradation becomes the sole mechanism responsible for TC reduction. Calculating the adsorption coefficient k_d (i.e., solid-water distribution coefficient), the equation of which is written in Appendix A (Carballa et al., 2008), yielded a value of 35083 L/kg. This gives an idea about the affinity of tetracycline to adsorb to anaerobic sludge get distributed among the solid phase and the aqueous phase based on the properties and factors to be discussed in the next chapter.

Figure 17 – Concentration of Tetracycline Adsorbed to Sludge Solids During Phase 2

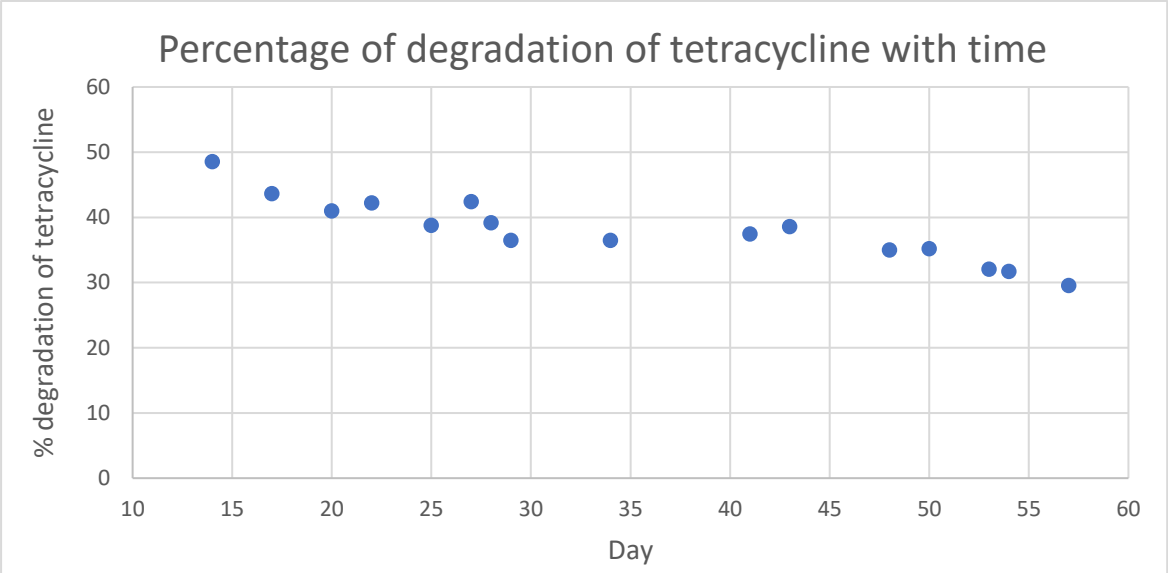


5.4 Evaluation of the Biodegradation of Tetracycline Based on a Mass Balance

Earlier, four pathways were pointed out for the elimination of OMPs by MBR processes which are (i) biotransformation or degradation (photodegradation or biodegradation); (ii) sorption onto the sludge; (iii) volatilization or stripping by aeration; and (iv) physical retention by membranes (Cirja et al., 2008; Li et al., 2015). Because volatilization of tetracycline at a temperature of 30 °C and the physical retention of tetracyclines in the MBR process is not probable as previously mentioned, these two mechanisms can thus be neglected leading to shedding light on degradation and sorption onto the sludge. Because a predetermined concentration of 300 µg TC/L was fed into the system, and because the tetracycline concentration in the three effluents and that adsorbed to sludge were evaluated, the percentage of degradation of tetracycline can thus be deduced by a simple mass balance presented in Appendix A. Figure 18 shows that within a few days after the start of phase 2 of the experiment, around 50% of the tetracycline fed into the system was degraded. With time, the percentage of degradation decreased because both the concentrations in the effluents and onto the sludge were increasing. During the span of phase 2, degradation decreased by ≈ 20% reaching a value of

29.57 % on day 57. Therefore, an average of 38.19 ± 4.66 % was evaluated for tetracycline degradation during phase 2 which can be remodeled in terms of the biological degradation constant k_{BD} computed to equal to 1.5128 $\frac{L}{gMLVSS.d}$ based on the formula presented in Appendix A and derived from Harb et al. (2021).

Figure 18 – Percentage of Degradation of Tetracycline During Phase 2



5.5 Evaluation of Tetracycline Adsorption to Biofilms Formed on the Three Membranes

A procedure was developed and implemented to extract and quantify tetracycline in both loose and tight layers of the UF and MF2 membranes harvested 20 days after the start of phase 2 and in all three membranes on the last day of the experiment (Table 2). The evaluated concentrations in µg TC/L were converted to µg/cm^2 membrane effective area for better representation and understanding. The results showed no tetracycline detection in the loose layer of all harvested membranes on both harvesting points; however, all tight layers embodied tetracycline at different quantities. For the MF2 and UF membranes harvested on day 30, the

UF membrane incorporated 0.66 µg TC/cm^2 within its tight biofilm layer, while 0.05 µg TC/cm^2 were quantified for MF2. On day 60, MF1, which wasn't replaced at any point from the very beginning of the experiment, integrated the least TC mass among the three membranes, evaluated at 0.13 µg TC/cm^2 in its tightly bound biofilm layer. MF2 membrane trapped 0.18 µg TC/cm^2 in a thirty-day span, while the UF membrane recorded the highest mass of 1.38 µg TC/cm^2. Even though the mass of tetracycline adsorbed to the biofilm was assessed, it can be neglected when setting up the equation of the mass balance of tetracycline in the system.

Table 2 – Tetracycline Adsorption to Biofilm Loose & Tight Layers

Biofilm layer	**Concentration on day 30 (µg/L)**	**Concentration on day 30 (µg/cm^2)**	**Concentration on day 60 (µg/L)**	**Concentration on day 60 (µg/cm^2)**
MF1 loose	-	-	0	0
MF1 tight	-	-	74.3	0.13
UF loose	0	0	0	0
UF tight	366.6	0.66	769.7	1.38
MF2 loose	0	0	0	0
MF2 tight	30.3	0.05	102.8	0.18

5.6 Tetracycline ARGs in Effluents Before vs. After Tetracycline Addition

Even though the scope of the experiment was limited to a ten-day pre-tetracycline phase and a fifty-day post-tetracycline phase, a pre-tetracycline phase that incorporates the experiment done by Master student at the Lebanese American University, Charbel Khoury, extending aforetime phase 1 was considered for better representation of the 'no tetracycline' stage in the quantification of ARGs. This would also disregard the effect of the factor of time in the

development of ARGs as we wouldn't be comparing ARG development within 10 days (phase 1) to it within a five times higher span of 50 days (phase 2). Figures 18 and 19 respectively show the average intracellular and extracellular tetracycline resistance genes in the three membrane permeates before and after the addition of tetracycline. The results show that tetracycline exposure caused an increase in the number of copies of the addressed intracellular *tet* genes in all three permeates (Figure 19), however, no notable change or consistent trend was observed for the extracellular *tet* genes (Figure 20). Only *tetW* genes in MF2 permeate demonstrated a decrease of less than one order of magnitude after tetracycline exposure. The increase in the concentration of intracellular *tet* genes varied between less than an order of magnitude (*tetW* and *tetQ*), to around three orders of magnitude (*tetA* in MF2 permeate). Speaking about extracellular *tet* genes, their erratic changes were generally insignificant in terms of magnitude. It is important to note that extracellular *tetQ* genes were not detected and magnified in all three permeates at any phase of the experiment.

Figure 19 – Average Concentrations of Intracellular Tetracycline Resistance Genes for the Three Membrane Permeates Before and After Tetracycline Addition

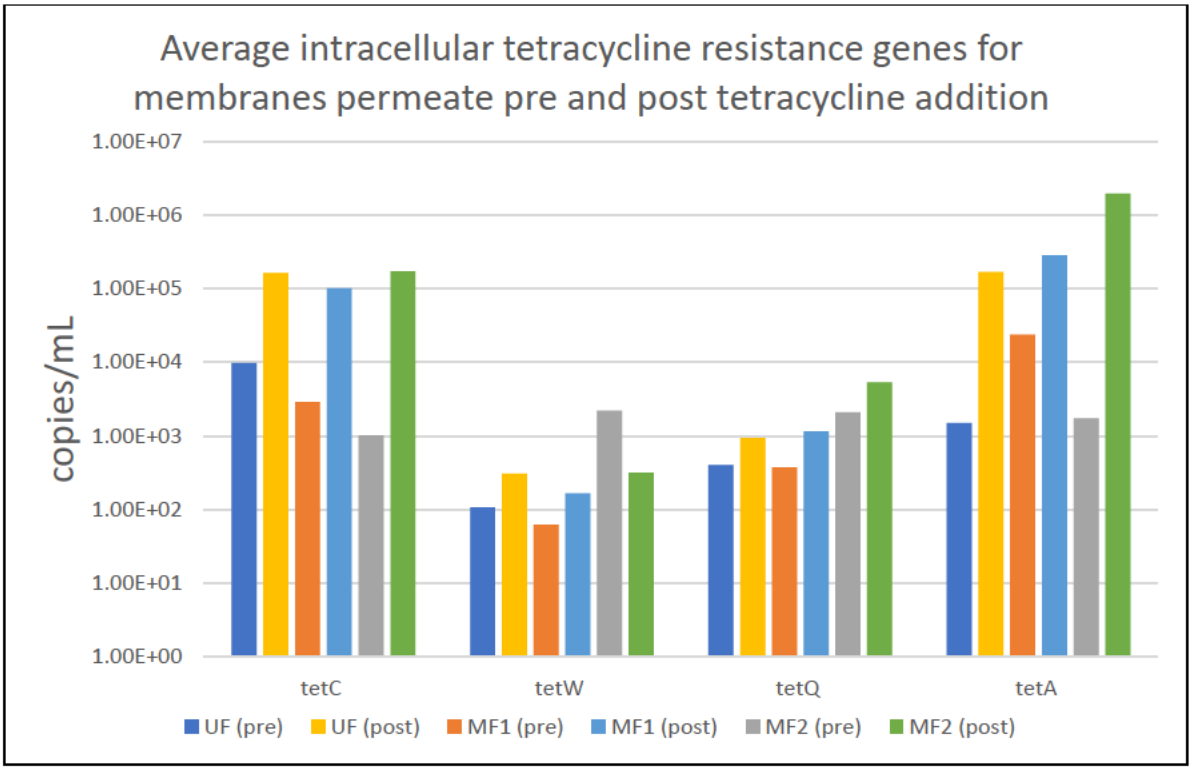
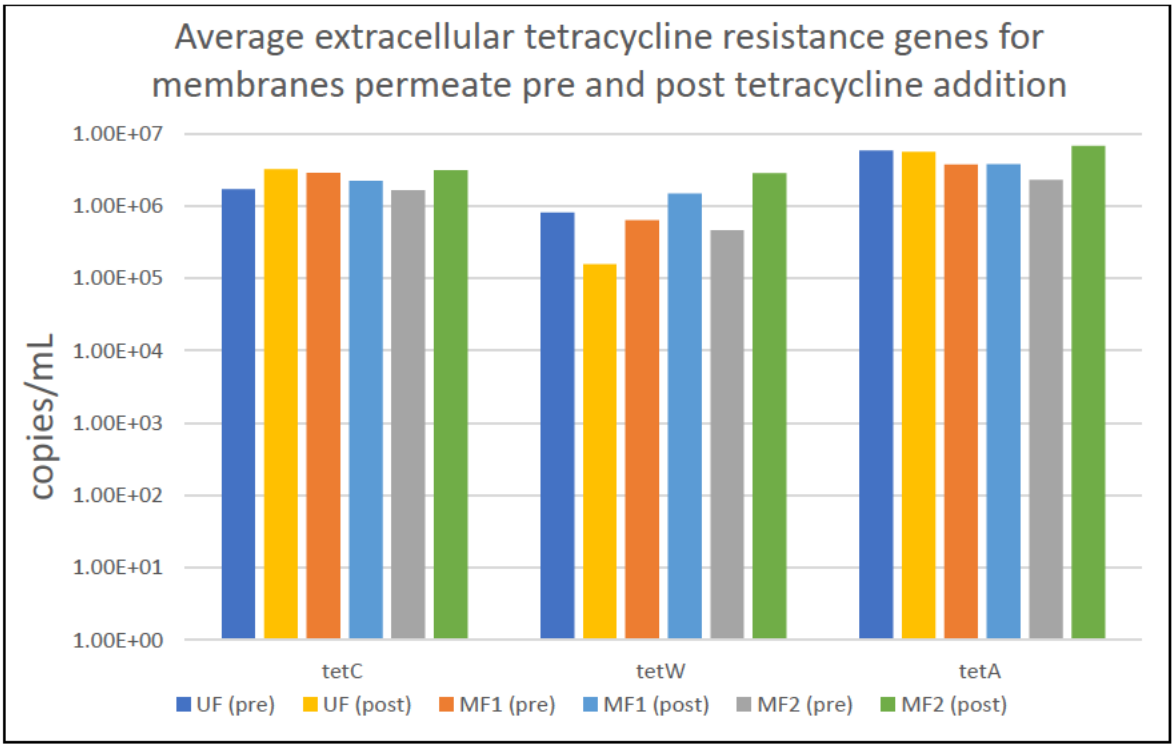


Figure 20 – Average Concentrations of Extracellular Tetracycline Resistance Genes for the Three Membrane Permeates Before and After Tetracycline Addition



5.7 Non-*tet* ARGs in UF and MF1 Effluents Before vs. After Tetracycline Addition

Regarding other four non-*tet* ARGs, they were investigated and compared in the two phases for the UF and MF1 effluents (Figures 20 & 21). The permeate of the MF2 membrane was not inspected in this sense due to the unrepresentative number of DNA extractions done during the pre-tetracycline phase and thus the inadequate number of data points we have. The results showed that each gene and form of its presence interacted differently with tetracycline exposure. For the intracellular ARGs (Figure 21), *intl1* and *sul1* concentrations increased by more than one order of magnitude (from 3.92E+06 to 6.61E+07 for UF and 4.50E+06 to 8.00E+07 copies/mL for MF1). *AmpC* showed a small decrease in the UF permeate in contrast to the small increase reported in the MF1 permeate. Nonetheless, this change is relatively insignificant to build a conclusion on. As for the *ereA* gene, tetracycline induced a minor decrease in its concentration in both examined effluents. Moving to extracellular ARGs (Figure 22), only *intl1* demonstrated a notable increase in concentration triggered by tetracycline presence (from 7.39E+07 to 4.67E+08 for UF and 6.72E+07 to 6.38E+08 copies/mL for MF1). As for *sul1*, *ampC*, and *ereA*, a decrease in the number of copies/mL is reported; however, this decrease is mostly significant for *ereA* demonstrated by a reduction of 5.79E+08 copies/mL and 6.41E+08 copies/mL for UF and MF1 respectively. It is important to note that whether in phase 1 or phase 2 of the experiment, there was negligible variation between the UF and MF1 membrane permeates in terms of the 4 aforementioned genes in both their intracellular and extracellular fractions of DNA. This brings about the lack of correspondence between the membrane pore size and the development of non-*tet* ARGs. Nonetheless, one limitation of what definite conclusion can be drawn from this is the difference in the age of biofilm formed on the surface of each of the membranes due to the fact that MF1 membrane was kept from an

experiment conducted prior to the start of our experiment, and was not harvested until the end of the experiment, while a new UF membrane was set up at the beginning of the experiment and harvested and replaced 20 days after the start of the tetracycline phase.

Figure 21 – Average Concentrations of Other Intracellular Resistance Genes for UF and MF1 Membrane Permeates Before and After Tetracycline Addition

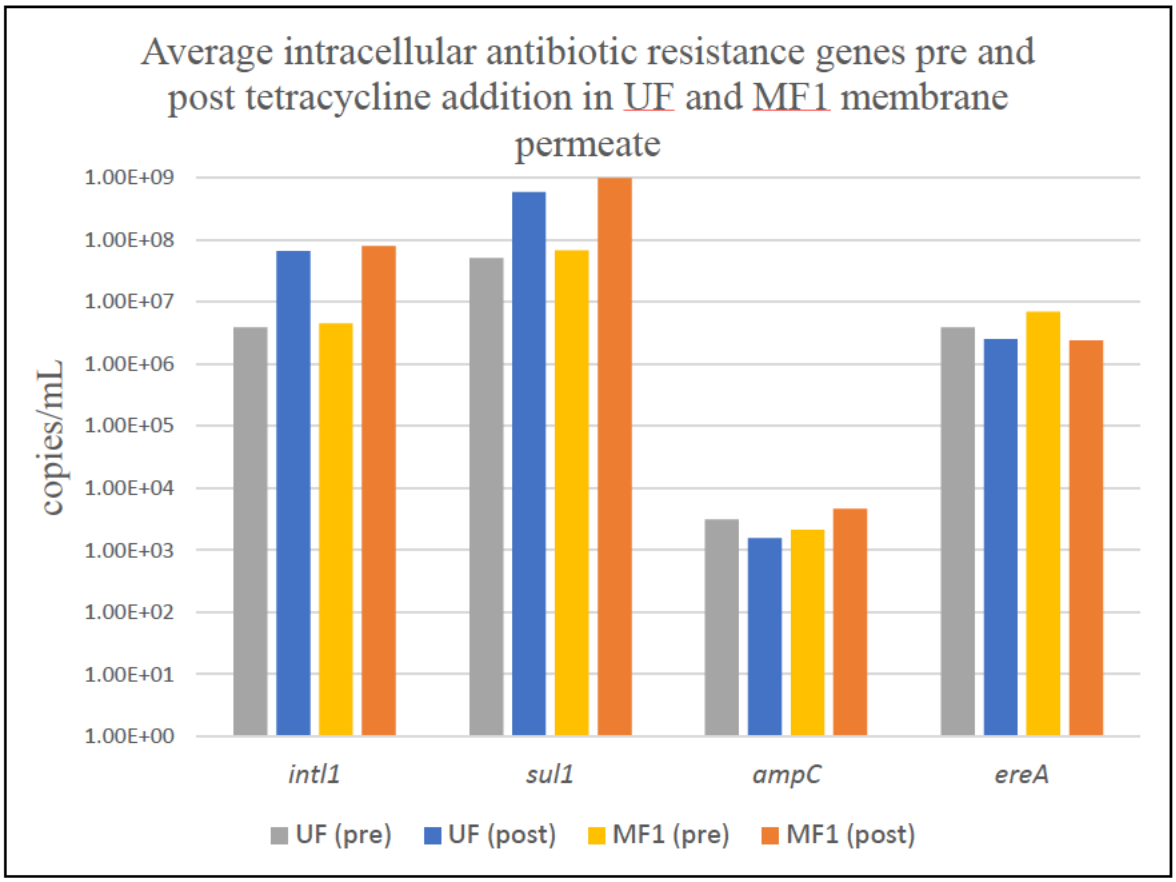
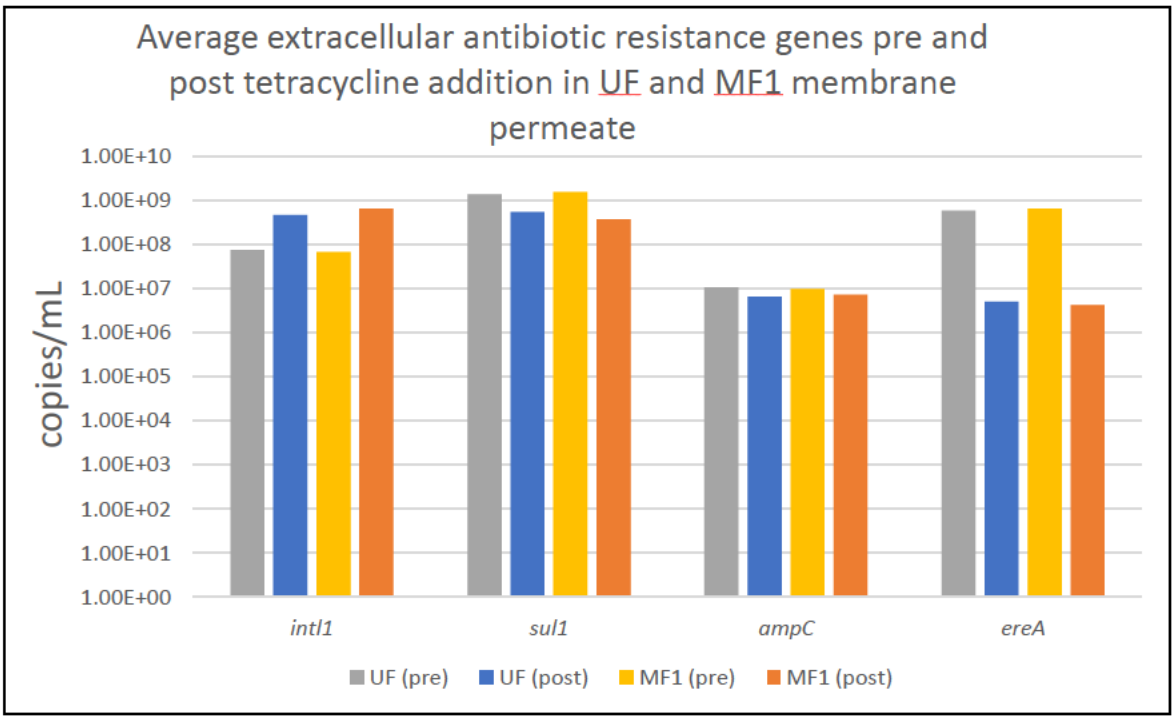


Figure 22 – Average Concentrations of Other Extracellular Resistance Genes for UF and MF1 Membrane Permeates Before and After Tetracycline Addition

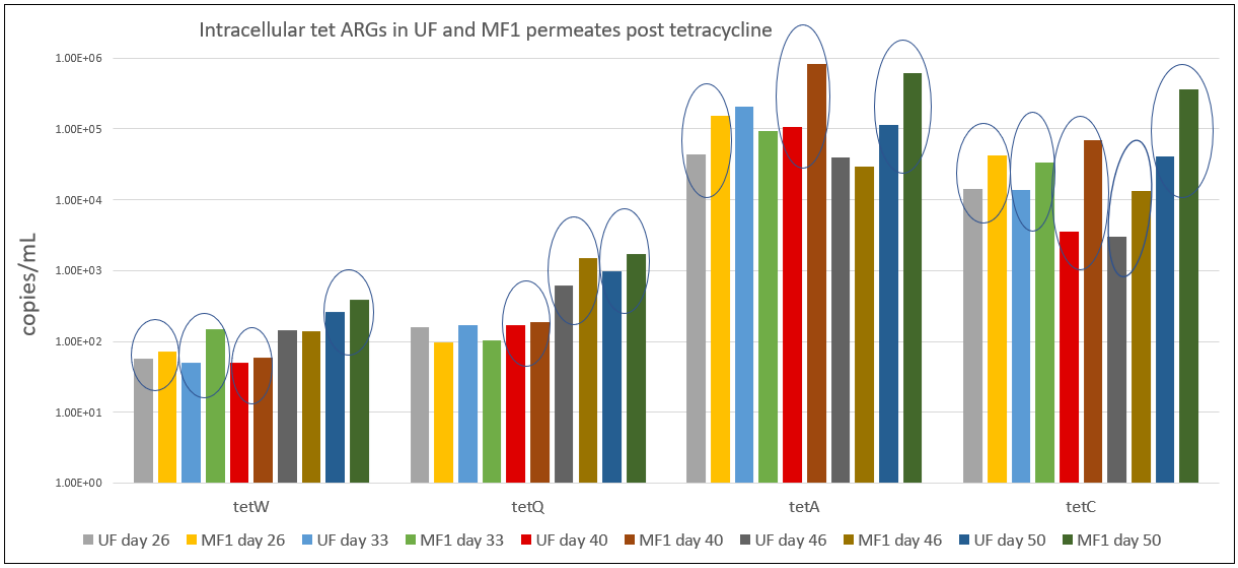


5.8 Tetracycline Intracellular ARGs in UF vs. MF1 Effluents After Tetracycline Addition

For the intent of comparing the concentration of tetracycline in the permeates of UF and MF1 for different sampling points throughout the tetracycline addition phase, the graph presented in Figure 23 was constructed. The results show that for the majority of the sampling points (15 out of 20), MF1 membrane permeate demonstrated a higher number of copies per volume for the four addressed tetracycline ARGs in comparison to UF membrane permeate. This implies the preference of UF membrane over MF membrane for retaining tetracycline resistance genes by the action of the nature, quantity, or quality of the microbial community that develops on the UF membrane surface. However, the same limitation of thought presented

in the previous section stands out which is the difference in the age of biofilm which could have brought about altered results.

Figure 23 – Concentration of Tetracycline Intracellular ARGs in the Effluents of UF and MF1 Membranes in Different Samplings Throughout Tetracycline Phase



CHAPTER SIX

DISCUSSION

The goal of the experiment and the target behind the derived results was not restricted to investigating the effect of tetracycline exposure on the performance of an AnMBR system treating municipal wastewater. Rather, addressing the efficiency of this system on tetracycline reduction, investigating the contribution of the multiple possible mechanisms of removal, and determining the effect of tetracycline on the development of intracellular and extracellular ARGs were the essence of the conducted research. To begin with, tetracycline addition caused a very minor disruption of the performance of the AnMBR which was manifested by a < 10 % drop in the COD removal and small deviation of the volume of methane produced from the expected; nevertheless, this disruption was quickly recovered within ≈ 5 days. This indicates that tetracycline almost caused no effect on the operational efficiency of the AnMBR; exposure to continuous concentration of 300 µg TC/L did not impose turmoil on the anaerobic digestion process performed by the microbial community signifying that this initial concentration is not high enough to induce an inhibitory effect on the activity of anaerobic microorganisms. This could be somehow linked to what Cetecioglu et al. (2014) figured out about tetracycline not affecting methanogens and archaea which explains the absence of a notable disruption in terms of the volume of methane produced (Figure 10). This conforms with previous studies, summarized by Cheng et al. (2018a), that scrutinized the treatment of wastewater containing different antibiotics at a wide range of concentrations by an AnMBR system and yet demonstrated high COD removal (> 84%).

As for the increase in the methane dissolved in each of the three effluents, UF and MF2 immediately and MF1 ≈ 20 days after the start of phase 2, literature reported that the operational

temperature of the system is what primarily influences CH_4 solubility in the liquid phase in an inverse manner (Crone et al., 2016; Giménez et al., 2012). However, since this is not the case here as the temperature was maintained at 30 °C throughout the entire experiment, this could be explained by an influence imposed by tetracycline on the microbial community. Microorganisms present in the system would generally alter the partitioning of produced methane between the liquid and the gaseous phases, and microorganisms attached to each membrane surface contribute to the variation noted among different membranes in this regard. Cheng et al. (2018b) discussed how the presence of antibiotics in AnMBRs influences fouling-related microbial communities besides other fouling-related factors such as sludge particle size, extracellular polymeric substances (EPS) and soluble microbial products (SMP) which could aggravate membrane fouling manifested through an increase in the TMP which itself is known to affect the permeability of methane through the membrane (Sanchis-Perucho et al., 2020). For tetracycline for instance, Zhu et al. (2018) identified that the addition of sulfamethoxazole (SMX) and tetracycline (TC) into the reactor each at 100 μg/L decreased the membrane fouling cycle from 25 days to 8 days which decreased further to 4 days when the concentration of SMX and TC was increased to 1000 μg/L.

Moving to tetracycline removal, the AnMBR showed very high efficiency yielding an average removal of 95.21 %, 91.81 %, and 95.70 % for MF1, UF, and MF2 membrane permeates respectively which is higher than what Liu et al. (2022) reported on the average removal of tetracycline by AnMBR evaluated at approximately 72%. UF membrane demonstrated a lower removal efficiency than both MFs which is likely attributable to the microbial community that conquers the membrane surface as UF has a lower pore size than MF which accentuates on the absence of any contribution for physical tetracycline retention, and both membranes are of the same material cancelling out the effect of varying tetracycline adsorption favorability due to material. Comparing the two MFs, no evident variation in terms

of TC removal was noticed which was then affirmed by the evaluation of TC in the membrane biofilms which almost show no difference. Lou et al. (2018) attributed the high removal of tetracyclines, particularly tetracycline and oxytetracycline to their amide and carboxyl groups to be readily absorbed onto biosolid and then biodegraded. This is congruent with the results we got in terms of high adsorption of tetracycline to sludge presented in Figure 17. As for degradation which was averaged to ≈ 38 %, it is close to what Cetecioglu et al. (2014) evaluated for biodegradation of tetracycline in a batch system under methanogenic conditions (46 %). The adsorption coefficient of tetracycline, k_d, the calculation of which is listed under Appendix A, is evaluated at around 35083 L/kg taking into consideration the averages of the last three recorded concentrations of each of tetracycline adsorbed onto the sludge and tetracycline identified in the effluent (highest concentration, i.e., UF membrane effluent) as the state of equilibrium. This is more than four times higher than what literature reported on the adsorption coefficient of tetracycline onto activated sludge ($k_d = 8400$ L/kg) (Kim et al., 2005; Wu et al., 2009). Even though USEPA (2007) reported the octanol–water partition coefficient log K_{ow} of tetracycline to be -1.3 which signifies a highly hydrophilic nature, surprisingly a high adsorption coefficient was determined which elicits the conclusion that log K_{OW} severely underestimates tetracycline sorption rates under various circumstances. With the intent to find an explanation to the unforeseen high adsorption capacity of tetracycline onto sludge, unfortunately, there was lack of literature on the partitioning of tetracycline in anaerobic systems; however, the adsorption of tetracycline appears to be widely variable among different sludge types. For instance, k_d of tetracycline is up to 20 times higher in municipal biosolids than in poultry solids (D'Angelo, 2017). Also, a wide range of k_d values between 0.47 and 8.4 L/g was reported for TC sorption on activated sludge (Kim et al., 2005; Plósz et al., 2010; Prado et al., 2009a). This implies that the characteristics of the sludge itself play a major role in how much they favor adsorption of tetracycline, and whether this is attributable to sludge content of

amino acids, proteins, polysaccharides, fatty acids, humic substances, or any other potential constituent of sludge is something that needs a particularized kind of investigation. It is also important to point out that the linear increase in TC adsorption to sludge means that the occupation of tetracycline on the surface of the solids and gradual decrease in the availability of free sites for adsorption did not impact the kinetics of the adsorption mechanism.

Regarding the contribution of biofilms to TC removal, the evaluated total mass of TC adsorbed to each of the three membranes connotes negligible tetracycline adsorption to the biofilm especially for the MF membranes. The microbial colonies that embody the UF membrane attract TC relatively higher than MF membranes, particularly for the tightly bound biofilm layer as the loosely bound layer did not exhibit any significance.

For the ARGs, we noticed that *tet*-associated iARGs in the effluent increased and were affected by membrane pore size. This accentuates the conclusion drawn by Wang et al. (2019) on the ramification of antibiotic exposure on the abundance of intracellular ARGs. The UF membrane demonstrated superior restraint of *tet*-asociated iARGs into the effluent in comparison to the MF1 membrane. However, *tet* eARGs did not show notable variation after tetracycline exposure, while other ARGs, in both their intracellular and extracellular forms of presence and in spite of their alternation of their concentration in response to tetracycline exposure, did not follow a consistent trend. Comparing *tet*-associated iARGs in the effluents of the MF1 versus UF could be attributed to difference in pore size or might be an accentuation on what Zarei-Baygi et al. (2020) demonstrated regarding the highly fouled membrane effluent having the highest absolute abundance of iARGs, which are *tet*-associated iARGs in our case.

CHAPTER SEVEN

CONCLUSION

The pollution of surface water and soil as a result of discharge or disposal of untreated or poorly treated wastewater has raised perilous concerns especially when the occurrence of antibiotics is involved and antibiotic resistance is considered. AnMBR has manifested various advantages over conventional wastewater treatment systems on one hand and aerobic MBR on the other, and it has been reported to be a promising technology for the removal of antibiotics and ARGs from wastewater. Thus, this experiment addressed the removal of tetracycline, which falls under one of the primary antibiotics groups used for veterinary, agricultural, aquacultural, and human therapeutic purposes and exhibits serious environmental problems, from municipal wastewater treated using a lab-scale AnMBR. The experiment which was composed of two phases, a pre-tetracycline phase and a tetracycline phase, aimed to examine the influence of tetracycline on the AnMBR performance generally and on tetracycline and *tet* and non-*tet* associated iARGs and eARGs particularly. The presented work showed that tetracycline continuously fed at a concentration of 300 µg/L did not disrupt the AnMBR efficiency, and it was removed at an average efficiency > 90% for all three membranes: a pre-acclimated MF membrane, a UF membrane, and a new MF membrane, though less efficient removal was recorded for the UF membrane. Tetracycline appeared to have high affinity to AnMBR biomass, which may have improved biodegradability. As for ARGs, *tet*-associated iARGs in the effluent increased and were affected by membrane pore size, whereas *tet* eARGs did not show notable variation after tetracycline exposure. Other (non-*tet*) ARGs also did not follow a consistent trend.

7.1 Future Work

Looking into the iARG and eARG profiles in the AnMBR for a post-tetracycline phase after which tetracycline stops being fed into the system hasn't been studied yet. This would be interesting to examine as it would allow to determine how the removal efficiency of tetracycline and the contribution of biodegradation gets altered beyond sludge full saturation. Also, a study on what and how sludge constituents and characteristics influence the extent of tetracycline adsorption would also be beneficial. Because the *tet*-associated iARGs became more abundant after tetracycline addition, post treatment strategies that primarily target ARG removal must be established during which horizontal gene transfer mechanisms and selectivity of ARGs and their form of existence are thoroughly studied.

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APPENDICES

APPENDIX A

Purified genes concentrations

The total number of copies for the purified genes was calculated using the formula:

$$Number\ of\ copies = \frac{Total\ concentration \times Avogadro's\ Number}{Length \times 10^9 \times Dalton's\ base\ pair\ weight}$$

Where,

Total concentration = total concentration of the purified gene per nanodrop (ng/µL)

Avogadro's Number = 6.023×10^{23}

Length = corresponding gene length (base pairs)

Dalton's base pair weight = 650

Expected methane production

$$0.25\ \text{g}\ CH_4/\text{g}\ COD_{\text{removed}} = \frac{0.25\ \text{g} \times 0.08205 \frac{L.atm}{mol.K} \times 298\ \text{K}}{16\frac{\text{g}}{mol} \times 1\ \text{atm.}} = 0.382\ \text{L}\ CH_4/\text{g}\ COD_{\text{removed}}$$

Expected CH_4 volume (L/d) = % COD removal × $COD_{influent}$ $(g/L) \times Q_{in}$ $(L/d) \times 0.382$

Effluent methane and supersaturation ratio calculation:

Volume of effluent sample collected in flask= 32 mL

Flask volume = 155 mL

Volume of biogas in bag = 50 mL

Methane volume in the headspace:

V= total headspace volume × % methane in headspace

V = (155-32+50) × % methane in headspace

Actual concentration of methane in the effluent:

$$C = \frac{V}{\text{effluent volume}}$$

$$C = \frac{173\ \text{mL} \times \%\ \text{methane in headspace}}{0.032}$$

Expected concentration of methane in the effluent:

Expected effluent methane concentration = % methane in reactor headspace × methane solubility

Knowing that at 35 ℃,

Methane solubility = 16.5 mg/L = 21.6 mL/L (Ideal Gas Law)

Expected effluent methane concentration = % methane in reactor headspace × 21.6 mL/L

Solid-water distribution coefficient (adsorption coefficient)

$$k_d = \frac{X}{S}$$

Where,

k_d = solid-water distribution coefficient

X = tetracycline concentration in the sludge (mg/kg TSS)

S = tetracycline concentration in the aqueous phase (mg/L)

Tetracycline mass balance

$$m_{influent} = m_{effluent,MF1,i} + m_{effluent,UF,i} + m_{effluent,MF2,i} + m_{sorbed,i} + m_{degraded,i}$$

Where,

$m_{influent}$ = mass of tetracycline in the influent = 300 µg/L

$m_{effluent,MF1}$ = mass of tetracycline in the effluent of membrane MF1 at sampling i

$m_{effluent,UF}$ = mass of tetracycline in the effluent of membrane UF at sampling i

$m_{effluent,MF2}$ = mass of tetracycline in the effluent of membrane MF2 at sampling i

$m_{sorbed,i}$ = mass of tetracycline adsorbed onto the sludge at sampling i

$m_{degraded,i}$ = mass of tetracycline degraded at sampling i

Biological degradation constant k_{BD}

$$\frac{c}{c_0} = \frac{1}{1+k_{BD}\theta X_{VS}}$$

Where,

c = tetracycline concentration in the effluent (µg/L)

c_0 = tetracycline concentration in the influent (µg/L)

k_{BD} = biological degradation constant (L/gMLVSS/d)

θ = HRT (d)

X_{VSS} = VSS concentration (g/L)

APPENDIX B

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

Where, slope = corresponding gene standard curve slope

Table 3 – qPCR Target Genes Amplification Efficiency

Gene	R^2	Amplification efficiency (%)	Amplification factor
intl1	0.9879	102	2.01
sul1	0.9998	93	1.93
ampC	0.9963	97	1.97
ereA	0.9995	80	1.8
tetA	0.9992	86	1.86
tetC	0.9996	89	1.89
tetQ	0.9987	87	1.87
tetW	0.9995	88	1.88

Normalization of DNA concentration

$$\text{Copies/mL} = \frac{\text{Copies yielded by qPCR(copies/100µL)} \times 100\ \mu\text{L}}{\text{Filtrate volume (mL)}}$$

Where,

100 µL = elution volume in the final step of the DNA extraction kit = volume of DNA obtained

Filtrate volume = volume of filtered influent or effluent