Antimicrobial Resistance in a Membrane Enhanced Biological Phosphorus Removal Process

by

Mehrnoush Mohammadali

B.Sc., University of Tehran, 2004

M.Sc, Tehran Azad University, Science and Research Branch, 2008

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

The Faculty of Graduate and Postdoctoral Studies

(Civil Engineering)

THE UNIVERSITY OF BRITISH COLUMBIA

(VANCOUVER)

February 2018

ABSTRACT

Coupling of membrane bioreactors with enhanced biological phosphorus removal (EBPR) processes increases the potential for low-yield sludge production by operating systems at long solids retention times (SRTs). However, biological foaming of anoxic mixed liquor in membrane EBPR (MEBPR) systems causes operational challenges (e.g. interference with the estimation of total suspended solids in the system). Hence, operating MEBPR systems by regular wastage of foam bacteria as a means to control SRT has gained increasing attention. The present research aims to evaluate the performance of MEBPR processes operating at long SRT (60 days) through estimation of the ARB release rates in foam, waste aerobic mixed liquor and effluent.

The results indicated that, while superior antibiotic resistant bacterial (ARB) removal was achieved by membrane filtration in MEBPR systems (SRT= 60 and 25 days (control)), the proportions of sulfamethoxazole and trimethoprim resistant bacteria among mixed liquor heterotrophs exhibited statistically significant elevations relative to the influent. It was also demonstrated that extended SRT operations could achieve similar total daily release rates of ARB (amoxicillin, sulfamethoxazole, tetracycline) compared to those of the 25 day SRT train. In detail, waste aerobic mixed liquor and foam were found to be the major contributors to ARB release in the 25 and 60 day SRT trains, respectively. Data also showed that operating MEBPR systems at 25 and 60 day SRTs could reduce the total ARB release rates relative to the ARB inflow rates. It was also observed that the parallel trains significantly increased the relative abundances of the *sul*1 gene (normalized to 16S rRNA gene) in mixed liquor compared to those of the influent.

Using an Illumina MiSeq platform, the taxonomic compositions of bacterial communities in influent and MEBPR environments were identified. Data analysis exhibited a shift in bacterial composition from influent to mixed liquor and then to treated effluent. A significant similarity was also found between mixed liquor and foam at the 25 day SRT train.

Finally, PCR-based examination and functional screening of the fosmid library revealed the presence of nine antibiotic resistance genes conferring resistance to four classes of antimicrobials in the foaming compartment of the MEBPR process.

LAY SUMMARY

While treating the municipal wastewater, disposal of excess biosolids needs to be carefully managed, one reason being the potential for the release of antibiotic resistant bacteria from wastewater treatment systems to the receiving environments. In the present research, the performance of a particular wastewater treatment system configuration (membrane enhanced biological phosphorus removal (MEBPR) process) was evaluated through estimating the release rates of antibiotic resistant bacteria (ARB) in the by-products of this system (i.e. effluent, mixed liquor and foam). The present project demonstrated that waste foam needs be considered as one of the potential reservoirs of ARB and antibiotic resistance genes and thus it requires safe disposal or reuse. In addition, this study suggests that indirect biological tests (e.g. bacterial counts) should be included in the list of routine monitoring parameters of membrane-treated effluent to guarantee the quality of effluent prior to discharge.

PREFACE

This statement confirms that the author of this thesis is the primary person responsible for the design and initiation of this research project with direct input from my supervisors, Dr. Eric Hall and Dr. Julian Davies. My thesis committee members, Dr. Don Mavinic and Dr. Loretta Li as well as Dr. Vivian Miao also made considerable contributions to the design of the research program. To assist with experimental set-up, Melanie Scofield in the Hallam Laboratory (Department of Microbiology and Immunology, Life Sciences Centre, UBC) provided initial training of laboratory protocols and assisted with filtering the effluent samples and their storage. Sam Kheirandish in the Hallam Laboratory provided initial training with the QPix2 Robotic colony picker, Varioskan Flash spectral scanning plate reader and CFX96 real-time PCR detection system. Mónica Torres-Beltrán kindly helped with the preparation of bubble plots and hierarchical cluster dendrograms using the R software. Christina Kim received training from me and assisted with filtering samples of membrane effluent as well as preparation of crude DNA lysates for 16S rRNA gene sequencing in the Davies Laboratory (Department of Microbiology and Immunology, Life Sciences Centre, UBC). Matthew Dickey helped with identification of ARGs in the *E. coli* EPI300:F3.18 library clone and antimicrobial screening of the foam fosmid library in the Davies Laboratory.

Excerpts from Section 3.2 were presented as a poster at the Life Sciences Institute (LSI) Research day held on March 11th, 2016, UBC, the Water and Environmental Student Talks (WEST) third annual conference held on June 7th, 2016, UBC and are in preparation for submission to a peer-reviewed journal.

Other publications during the PhD program includes the following:

1. Osachoff, H.L., Mohammadali, M., Skirrow, R.C., Hall, E.R., Brown, L., Van Aggelen, G.C., Kennedy, C.J., and Helbing, C.C. (2014) Evaluating the treatment of a synthetic wastewater containing a pharmaceutical and personal care product chemical cocktail: Compound removal efficiency and effects on juvenile rainbow trout, 2014. *Water Research* **62**: 271-280.

2. Mohammadali, M. & Davies, J. (2017) Antimicrobial resistance genes and wastewater treatment. In Keen, P. & Fugère, R. (Editors) Antimicrobial resistance in the wastewater treatment process. Wiley-Blackwell, Hoboken, N.J.

The content of this chapter is presented with minor changes in Section 1.2 of the present dissertation.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

ADP	Adenosine diphosphate
AMC	Amoxicillin/clavulanic acid
AMP	Ampicillin
AMX	Amoxicillin
AMK	Amikacin
AR	Antimicrobial resistance
ARB	Antibiotic resistant bacteria
ARG	Antibiotic resistance gene
ATP	Adenosine triphosphate
AZM	Azithromycin
AU	Approximately unbiased
BLAST	Basic local alignment tool
BP	Bootstrap probability
CM	Chloramphenicol
CARB	Carbenicillin
CFM	Cefixime
CFU	Colony forming unit
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
COD	Chemical oxygen demand
CsCl	Cesium chloride
COL	Colistin
CV	Coefficient of variation
DAP	Daptomycin
ddH_2O	Distilled deionized water
DEW	Distant early warning
DGGE	Denaturing gradient gel electrophoresis
DHFR	Dihydrofolate reductase

DHPS	Dihydropteroate synthase
DNA	Deoxyribonucleic acid
DOX	Doxycycline
EDTA	Ethylenediaminetetraacetic acid
EMB	Eosin methylene blue
EMP	Embden–Meyerhoff–Parnas
EPS	Extracellular polymeric substance
EtBr	Ethidium bromide
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FISH	Fluorescence in situ hybridization
G(+)	Gram-positive
G(-)	Gram-negative
GAO	Glycogen-accumulating organism
GEN	Gentamicin
HGT	Horizontal gene transfer
HRT	Hydraulic retention time
HWW	Hospital wastewater
IMP	Imipenem
IS	Insertion sequence
IUPAC	International Union of Pure and Applied Chemistry
KAN	Kanamycin
Lac+	Lactose fermenting
Lac-	Non-lactose fermenting
LB broth	Luria Bertani broth
LPS	Lipopolysaccharide
MBR	Membrane bioreactor
MDR	Multidrug-resistant/Multi-drug resistance
MEBPR	Membrane enhanced biological phosphorus removal
MGE	Mobile genetic element
MH	Müeller-Hinton
MIC	Minimum inhibitory concentration

MRSA	Methicillin^{R} *Staphylococcus aureus*
MUP	Mupirocin
N	Nitrogen
NAL	Nalidixic acid
NADH	Nicotinamide adenine dinucleotide+Hydrogen
NaOAc	Sodium acetate
NAPS	Nucleic Acid Protein Service Unit
NCBI	National Centre for Biotechnology Information
OD	Optical density
ORF	Open reading farme
OTU	Operational taxonomic unit
P	Phosphorus
PABA	Para-aminobenzoic acid
PAO	Polyphosphate-accumulating organism
PBS	Phosphate buffered saline
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PFGE	Pulse field gel electrophoresis
PHA	Polyhydroxyalkanoate
PL-B	Polymyxin B
Poly P	Polyphosphate
qPCR	Quantitative PCR
R2A	Reasoner's 2A
RIF	Rifampicin
RNA	Ribonucleic acid
STR	Streptomycin
SBR	Sequencing batch reactor
SCFA	Short chain fatty acid
SLB	Sucrose lysis buffer
SMX	Sulfamethoxazole
SPC	Spectinomycin

SRT	Solids retention time
SSCP	Single strand conformation polymorphism
TAE	Tris acetate EDTA
TCA	Tricarboxylic acid
TET	Tetracycline
TGGE	Temperature gradient gel electrophoresis
TKN	Total Kjeldahl nitrogen
TMP	Trimethoprim
TP	Total phosphorus
Tris EDTA buffer	TE buffer
TSS	Total suspended solids
UBC	University of British Columbia
UCT	University of Cape Town
UV	Ultraviolet
VAN	Vancomycin
WWTP	Wastewater treatment plant

Acknowledgements

I would like to express my gratitude to my supervisor Dr. Eric Hall for all his guidance, thoughtful advice, patience and encouragement during the several years of my studies at UBC. Dr. Hall, thank you very much for supporting me all these years to transfer from Masters to the PhD program and then pursue my degree at the interface of environmental engineering and microbiology. Without your trust on me, I could never imagine this goal could ever be achieved.

I would also like to acknowledge my co-supervisor Dr. Julian Davies for his unwavering enthusiasm and thoughtful advice. Dr. Davies, I am very much honoured to be given the opportunity to work in your lab for several years. Your enthusiasm in research and hard work is exemplary. I will never forget the quiet Saturday and Sundays that you came to do some lab work, turning the light on and asking me “Do you really like to work in the dark?”

I would also like to thank my committee members Dr. Don Mavinic and Dr. Loretta Li for all their useful comments in preparation of this dissertation. I would also like to thank Dr. Steven Hallam for all his support and giving me the chance to perform a great portion of my experiments in his laboratory.

I am very appreciative of Dr. Vivian Miao for all her encouragement during the most difficult experiences. I truly cherish the many long conversations we had in the Davies Lab. Each session was truly a learning opportunity for me. I will miss your mentoring. Your passion for research is exemplary and not forgettable.

Many thanks also go to Dr. Patricia Keen for being such a good friend. I will never forget those very hard days that you were listening to my uncountable complaints and giving me the hope that I can make it through.

I am indebted to the members of Davies Lab especially our lab manager Iván Villanueva and my special friend Shekooh Behroozian. Thank you so much Ivan for providing such a safe and enjoyable work environment in Davies Lab. Shekooh, I am very glad to have met you in

my life. You tought me how the strong women survive the very hard days only with the magic of patience and faith.

My colleagues in the Environmental Engineering department, Hallam Lab and Gaynor Lab, Soubhagya, Alireza, Zaki, Sepideh, Marcia, Rony, Sahar, Monica and Martha, thanks for your friendship and support. I also appreciate help from Doriane, Mateos, Christina and Helena in my research work.

I would like to acknowledge National Sciences and Engineering Research Council of Canada (NSERC) for the financial support provided for this doctoral program.

To my sister (Maya), my niece (Sophie) and my brother in law (Reza) for all your love and support in my life and my studies. Maya, you know that just without your love, I could not move forward. Thanks for being such a wonderful sister.

To my husband (Nima) and my daughter (Nora) who was born in the middle of my PhD journey. I don't know how to thank both of you for making my life such colorful. Nora, I owe you so many hours of playing, so many weekends and so many kisses. I promise to make it up for you. Mommy loves you more than anything.

Finally, to my father (Mohammad) and my mother (Nasrin), words are unable to explain what I feel for you. Joon Joon, your love and support is the miracle of my life.

Dedication

In memory of Frederic Koch

1 INTRODUCTION AND LITERATURE REVIEW

1.1 Antimicrobials and Mechanisms of Resistance

As an important group of pharmaceuticals, antimicrobials are a class of naturally-occurring, semi-synthetic and synthetic organic molecules that are used to treat all manner of bacterial infections (Davies and Davies, 2010). There are several schemes for classifying antimicrobials, based on their chemical structures, mechanisms of action and range of effectiveness and cell death or growth suppression properties. Major structural classes of antimicrobials include β-lactams, macrolides, quinolones, tetracyclines, aminoglycosides, sulfonamides, pyrimidines and peptides (Morar and Wright, 2010). Based on their mode of action, antimicrobials are classified to inhibitors of (1) cell wall synthesis, (2) cell membrane function, (3) Deoxyribonucleic acid (DNA) synthesis, (4) protein synthesis and, inhibitors of other metabolic processes (metabolic antagonist action) such as folic acid pathways (Tenover, 2006; Fernandes et al., 2013). **Table 1.1** summarizes the modes of action of the major classes of antimicrobials.

According to the range of effectiveness, antimicrobials are divided into narrow and broad spectrum agents. For example, tetracyclines, as broad spectrum antibiotics, are effective against a wide range of Gram-negative (G-) and Gram-positive (G+) bacteria (Chopra and Roberts, 2001). Antimicrobial agents are also classified as bactericidal and bacteriostatic. Bacteriostatic antibiotics interfere with bacterial cellular mechanisms and prevent cell division; however, bactericidal antibiotics kill the bacteria, by damaging the cell membrane and causing leakage of cell contents (Bernatová et al., 2013).

While antibiotics have improved the quality of life worldwide, the development of bacterial resistance to a wide range of antibiotics has become a global public health issue (Nagulapally et al., 2009; West et al., 2011). Apart from natural resistance in which inherent structural or functional characteristics of certain bacterial species result in resistance to particular classes of antimicrobials, acquired antimicrobial resistance (AR) occurs by chromosomal mutation or acquisition of antibiotic resistance genes (ARGs) carried by mobile genetic elements (MGEs) (Hollenbeck and Rice, 2012; Munita and Arias, 2016).

Mechanisms of AR involve enzymatic inactivation and degradation of antibiotics, alteration in antibiotic target site, enhanced efflux of antibiotics through efflux pumps, impaired uptake of antibiotics and substitution of antibiotic insensitive targets (**Table 1.1**) (Levy, 1992).

Table 1.1 Antimicrobials modes of action and mechanisms of bacterial resistance to major classes of antimicrobials (adapted from Davies and Davies, 2010)

Antimicrobial class	Example(s)	Target	Mechanism(s) of Resistance
β-lactams	penicillins (e.g. amoxicillin (AMX)) cephalosporins (e.g. cefixime (CFM)) penems (e.g. imipenem (IMP))	cell wall (peptidoglycan biosynthesis)	hydrolysis, altered target, efflux
Glycopeptides	vancomycin (VAN)	cell wall (peptidoglycan biosynthesis)	reprogramming peptidoglycan biosynthesis
Lipopeptides	daptomycin (DAP)	cell membrane	altered target
Cationic peptides	colistin (COL)	cell membrane	altered target, efflux
Aminoglycosides	amikacin (AMK) spectinomycin (SPC) gentamicin (GEN) kanamycin (KAN)	protein synthesis (messenger-ribonucleic acid (m-RNA) translation)	enzymatic modification (acetylation, phosphorylation, nucleotidylation) efflux, altered target
Tetracyclines	tetracycline (TET)	protein synthesis (mRNA translation)	monooxygenation, efflux, altered target
Macrolides	azithromycin (AZM)	protein synthesis (mRNA translation)	hydrolysis, glycosylation, phosphorylation, efflux, altered target
Rifamycins	rifampicin (RIF)	protein synthesis (transcription)	adenosine diphosphate (ADP)-ribosylation, efflux, altered target
Quinolones/ Fluoroquinolones	ciprofloxacin (CIP)	DNA replication	acetylation, efflux, altered target
Sulfonamides	sulfamethoxazole (SMX)	folic acid synthesis (conversion of para-aminobenzoic acid (PABA) to dihydropteroic acid)	efflux, altered target (dihydropteroate synthase (DHPS) enzyme)
Pyrimidine	trimethoprim (TMP)	folic acid synthesis (conversion of dihydrofolic acid to tetrahydrofolic acid)	efflux, altered target (dihydrofolate reductase (DHFR) enzyme)

Genetic elements such as self-transmissible plasmids and associated elements (conjugative

transposons, integrons and insertion sequences (ISs) can be acquired through conjugation, transduction and transformation (Tennstedt et al., 2003). Conjugation, the major mechanism of horizontal gene transfer (HGT), occurs by transferring bacterial DNA between cells (of even different genera of donor and recipient) that are in physical contact (Usha et al., 2010). The transduction involves gene transfer via bacteriophages (Balcazar, 2014). Transformation is a process in which a recipient cell takes up genetic material from an external environment such as dead bacterial cells (McGee et al., 2001). Once ARGs are acquired, the genes encoding these defence mechanisms are transmitted directly to the bacterial progeny via cell replication.

1.2 Antibiotic Resistance Genes and Wastewater Treatment*

Since ancient times, the human population has randomly disposed of its waste into the environment, such as rivers and cesspits. The industrial revolution of the late 18th and early 19th centuries was a period that serves as a prime example of disposal of toxic organic chemicals by direct release into the environment. Many of these toxic molecules had antimicrobial activity, and it can be assumed that microbes resistant to these toxins multiplied in such environments. As a modern example, one can cite the concentrations of heavy oils that were dumped near detection stations in the distant early warning (DEW) line at the end of the Second World War. These sites are now excellent sources of bacteria with enhanced biodegradation capacities and they have been extensively studied in recent years (Slater et al., 2011; Bolton, 2012).

Following the discovery of the chemically-synthesized sulfonamides and trimethoprim (TMP) and the identification of dual resistance in 1969, the subsequent and most disastrous environmental pollution has come from the disposal of antibiotic production wastes in various forms. These discarded products were developed as food supplements for farm animals and their use became common practice for promoting weight gain in all aspects of

* The content of Section 1.2 appears in "Mohammadali, M. & Davies, J. (2017) Antimicrobial resistance genes and wastewater treatment. In Keen, P. & Fugère, R. (Editors) Antimicrobial resistance in the wastewater treatment process. Wiley-Blackwell, Hoboken, N.J." with minor modifications.

animal and fish husbandry worldwide. The amounts of antibiotics and antibiotic wastes disposed of in this way cannot be accurately determined. However, according to recent estimates by the Union of Concerned Scientists in the United States, antibiotic use for non-therapeutic purposes in three major livestock sectors (chickens, cattle, and swine) was about eight times more than the consumption for human medicine (Mellon et al., 2001).

In the past 50 years or so, the world has been faced with the rapid evolution of antimicrobial resistance (AR). Strachan and Davies (2016) stated, "Although AR is not considered as a disease in itself, it causes the failure in effective prevention and treatment of many diseases and greatly increases the rate of morbidity and mortality". The global use of antibiotics at low cost, auto medication and short duration of treatment has accelerated, extended and expanded the spectra of resistance worldwide. The earth has been continuously bathed in a dilute solution of antibiotics for more than half a century.

Aquatic ecosystems have been identified as hotspots of resistance mechanisms (Rizzo et al., 2013). This is due to the large diversity of pathogenic and commensal microorganisms and the continuous discharge of antibiotic resistant bacteria (ARB) and genes into these environments. As part of the aquatic environment, urban wastewater treatment systems (collecting sanitary sewage, hospital effluents and storm water runoff) possess all the components required to ensure the acquisition of all varieties of resistance genes. The antimicrobials present in wastewater due to incomplete degradation by humans and animals, disposal of unused drugs and runoff losses from land application, together with environmental and pathogenic bacteria in nutrient-rich engineered systems, provide all the necessary requirements to support a breeding ground for HGT and the propagation of resistance genes (Davies and Davies., 2010; Ferreira da Silva et al., 2006; Kim and Aga, 2007; Lefkowitz and Duran, 2009).

For about a century after the first biological wastewater treatment plant (WWTP) was built in Worcester, Massachusetts in 1890, many advances in wastewater treatment technology have improved the removal efficiencies of biodegradable organic pollutants (Metcalf and Eddy, 2003). Currently, membrane enhanced biological phosphorus removal (MEBPR) processes not only enable removal of traditional carbonaceous contaminants but also reduce P concentrations to very low levels (< 0.1 mg/L) in the effluent discharge (Zuthi et al.,

2013).

Over the past 15 years, increasing attention has shifted towards the identification of removal mechanisms of micropollutants from wastewater and sludge. Micropollutants are persistent organic or mineral substances such as personal care products, pharmaceuticals, pesticides and detergents whose discharge, even at very low concentrations, leads to constant growing environmental contamination (Luo et al., 2014).

Despite the evolution of wastewater treatment technologies from conventional to advanced treatment configurations, existing urban biological wastewater treatment systems are not specifically designed to remove micropollutants and ARGs. Studies on antibiotics as emerging classes of micropollutants have confirmed the high frequency of AR genotypes as well as ARB in wastewater treatment systems including constructed wetlands and WWTPs (Martins da Costa et al., 2006; Kim et al., 2010; Volkmann et al., 2004; Luczkiewicz et al., 2010; Reinthaler et al., 2003).

In a landmark series of papers published between 2003 and 2009, Szczepanowski and coworkers (2009) presented the first extensive DNA sequence-based screening of a large set of known ARGs in samples of activated sludge and the final effluent of a WWTP in Bielefeld-Heepen, Germany. This comprehensive survey identified 140 different clinically relevant AR genotypes and contaminants. From these investigations, it is evident that such treatment systems may play important roles in the development of multidrug-resistant (MDR) bacteria among complex microbial populations.

The occurrence of ARGs and ARB in the two main by-products of wastewater treatment systems (excess biosolids and treated effluent) has been reported frequently. Currently, effluent water quality standards, prior to discharge, are usually limited to controlling the concentrations of total residual chlorine, suspended solids, carbonaceous biochemical oxygen-demanding matter and un-ionized ammonia. There exist no regulatory guidelines to monitor and control the levels of ARGs in bacteria and extracellular DNA from lysed microbial cells in the effluent discharge. Studies have reported that AR determinants and MDR pathogens are transported with treated effluent to receiving waters (Iwane et al., 2001; Galvin et al., 2010; Goñi-Urriza et al., 2000). For example, LaPara et al (2011) showed that

the quantities of three tetracycline (TET) resistance genes were significantly higher in a tertiary treated effluent discharge than in receiving water samples in the Duluth-Superior Harbor, St. Louis River and Lake Superior, USA.

Despite the evidence for the occurrence of resistance genes in effluent discharges, the overall impact of treated wastewater applications on irrigation processes is unclear. Some studies have observed an increase in soil microbial activity and biomass after irrigation by treated wastewater as shown by a shift in the composition of soil bacterial communities (Oved et al., 2001; Broszat et al., 2014). However, recent studies have reported no major impact on AR in the wastewater-irrigated soil microbiome (Gatica and Cytryn, 2013; Negreanu et al., 2012).

The presence of ARB and ARGs in wastewater biosolids-amended soils is well documented (Brooks et al, 2007; Rahube et al., 2014). Biosolids are the treated and stabilized nutrient-rich organic residuals produced as a by-product of wastewater treatment which are widely used as fertilizer to simulate plant growth (Lu and Stoffella 2012). Recent studies have demonstrated that complementary technologies such as aerobic digestion and lime stabilization can be used as approaches to reduce the quantities of ARGs in biosolids (Munir et al., 2011). However, ARG concentrations and corresponding decay rates can be variable depending on the application methods, biosolids treatment reactor design, storage conditions, the specific ARGs involved and the frequency of biosolids application (Burch et al., 2013; Miller et al., 2014).

Although ARB and genes encoding AR have been commonly detected in wastewater and the by-products of treatment systems, the role of wastewater treatment processes in the development of AR is not clear. In recent years, a number of studies have investigated the variables affecting the patterns of ARB and ARGs in treatment processes (Xia et al., 2012; Yuan et al., 2014). However, even though many studies have reported a contribution from treatment processes to the evolution, spread and positive selection of antimicrobial resistant isolates, it also has been shown that wastewater treatment processes can act as efficient barriers to decrease the number of ARB and concentrations of ARGs (Gao et al., 2012; Duong et al., 2008; Nagulapally et al., 2009). The reasons for such discrepancies are the large number of variables in conditions such as influent source and input quality, treatment

process configurations and the process operating conditions.

Hospital wastewater (HWW) is likely to be a major contributor to the spread of pathogenic MDR bacteria in WWTPs (Brown et al., 2006). Due to the presence of constant sub-inhibitory levels of broad spectrum antimicrobials, hospital sewage creates a perfect situation for the exchange of ARGs and their combinations between clinical pathogens and environmental bacteria (Amador et al., 2015; Santoro et al., 2015). In this respect, the proportions of influent wastewater originating from institutions (including hospitals), blackwater (excreta, urine and faecal sludge), gray water (kitchen and bathing wastewater), stormwater and other urban runoff sources are important determinants of the input quality, the frequency of detection of ARGs and pathogenic ARB and the dissemination of antibiotics and AR from treatment plants (Harris et al., 2013).

Over the past few years, some European countries have constructed specialized WWTPs to provide separate treatment of HWW. Membrane bioreactor (MBR) technology as a pre-treatment, ozonation, powdered and granulated activated carbon have been proposed as the most attractive options to remove micropollutants from HWW (Beier et al., 2010; Beier et al., 2012; Kovalova et al., 2013). Very recently, Chonova and coworkers (2016) published a comparative study on the efficiency of the removal of antimicrobials from parallel wastewater systems providing separate treatment of hospital and urban wastewater. Despite the higher concentrations of antibiotics in the hospital influent as well as treated effluent, the results indicated increased removal efficiency of antibiotics during the separate treatment of HWW. It was also demonstrated that biofilm communities receiving hospital treated effluent had lower bacterial diversity and less developed biomass. Observations from this study confirm the adaptations of wastewater bacterial communities receiving HWW. With respect to the dedicated treatment of hospital waste, more studies are needed to reveal the mechanisms by which adapted biofilm microbial communities can be transferred to aquatic environments.

Advanced wastewater disinfection technologies such as ultraviolet radiation and ozonation are effective approaches to decrease the extent of ARB and levels of ARGs (Zhang et al., 2015a). However, other research has observed higher survival rates of resistant strains compared to sensitive bacteria, selection of ARGs and shifts in bacterial population in the effluent after advanced treatments (Lüddeke et al., 2015; Alexander et al., 2016; Hu et al.,

2016). Differences in reports on the efficiency of advanced approaches to wastewater treatment in controlling AR may be due to underestimates of variable operating conditions.

Solids retention time (SRT) is a design and operational parameter that has a crucial impact on the performance of activated sludge wastewater treatment processes. SRT, or the mean cell residence time, is defined as total solids mass present in the system divided by solids mass disposed of per day (Clara et al., 2005). As SRT controls the net growth rate of the entire system, it is the main factor influencing the composition of a wastewater microbial community (Benefield and Randall, 1980, Xia et al., 2012). As an example, Liu and Wang (2014) showed that the nitrite-oxidizing bacteria/ammonia-oxidizing bacteria ratio is considerably influenced by variations in SRT.

A recent approach to wastewater management minimizes biosolids production through microbial predation and metabolic changes (Amanatidou et al., 2015; Peccia and Westerhoff, 2015). One of the key factors which influences bacterial ecosystem manipulation and reduces excess production of sludge is operation of the system at high SRTs (Yoon et al., 2004; Li and Wu, 2014). However, the role of prolonged SRT on the composition of bacterial processes contributing to AR is not yet clear. Although antibiotic degradation is maximized by prolonged cell residence time, extended exposure of bacteria to antibiotics from the source may increase the potential for development of AR (Walston, 2013; Xia et al., 2012). Meanwhile, environmental concerns associated with transformation of antibiotics into other biologically active compounds during the extended SRT operations have not been considered in many cases. More detailed research is required to detect the antimicrobial degradation products in these treatment processes and to investigate the optimal SRT required to achieve the best ARG removal.

Another serious challenge in wastewater management is the control of filamentous bulking and foaming. Although filamentous microorganisms support activated sludge floc formation, their overabundance in WWTPs causes considerable operational difficulties such as poor sludge settling and thickening (Cydzik-Kwiatkowska et al., 2016; Pal et al., 2014). Different strategies have been employed to control foaming including polymer addition, the application of disinfectants such as chlorine, and the use of foam-classifying selectors to skim and remove foam bacteria (Parker et al., 2003). The use of bacteriophages to reduce the titer of

filamentous bacteria is one of the most promising environmentally-friendly approaches to control foaming (Liu et al., 2015). Despite the role of foaming bacteria on the efficiency of the treatment process and the environmental risks associated with foam disposal or formation of undesirable chlorinated by-products, no studies of AR patterns in foam-causing bacteria have been reported. More detailed studies of the impact of chemical disinfectants on the susceptibility profiles of foam-causing bacteria and their survival and gene transfer after disposal of resistant foam bacteria are needed.

For decades, culture-dependent approaches have been the most common methods used to study AR in WWTPs (Al-Bahry et al., 2009; Okoh and Igbinosa, 2010). In these studies, resistance profiles of pathogenic population subsets of bacterial communities downstream of the effluent discharge were studied. (Lefkowitz and Duran, 2009; Akiyama and Savin., 2010; Zhang et al., 2009).

Culture-dependent methods have also been used to investigate the role of mobile genetic elements (MGEs) in the dissemination of ARGs in WWTPs. It has been shown that MGEs influence bacterial evolution, adaptation and the roles that genetic elements play in the emergence, recombination and propagation of AR (Jackson et al., 2011). Studies to date have documented the incidence of integron-associated ARG cassettes on MGEs such as plasmids in WWTP samples (Tennstedt et al., 2003; Koczura et al., 2012; Kotlarska et al., 2015; Ma et al., 2011). In this respect, understanding the correlation between the distribution of pathogenic bacteria and associated integron patterns will aid in clarifying AR mechanisms in WWTPs.

In addition to resistance genes and associated elements, it is probable that virulence and biodegradation gene clusters are propagated in WWTPs. In a recent publication, Olaniran and colleagues (2015) detected four virulence-associated genes in *Listeria* and *Aeromonas spp*. isolated from treated effluents of two WWTPs and receiving waters in Durban, South Africa. Observations from this study emphasize the need for more investigation of virulent bacteria found in WWTPs and the co-occurrence of virulence genes and ARGs.

Despite the advantages of culture-dependent techniques, including low cost and the potential for combination with other methods, the availability of culture-based methods for

studies of environmental microbes gives a highly restricted view of microbial community structure in environmental ecosystems (Mahmod 2014). In this respect, the application of targeted and sequence-based metagenomics provides more extensive and accurate assessments of the abundance of ARGs and the phylogenetic and functional diversity of a wastewater resistome relative to the culture-based approaches (Wang et al., 2013; Schmieder and Edwards, 2012; Parsley et al., 2010; Ma et al., 2016). As an example, Wang and coworkers (2013) performed a metagenomic study of MGEs and ARGs in both anaerobic and aerobic sludge of a tannery WWTP in China. Metagenomic analyses showed that the taxonomic classification, as well as the abundance of functional genes in the aerobic and anaerobic sludge microbial communities, were different. They also observed a high prevalence of insertion sequences (ISs) and integrase genes highlighting the important role of MGEs in gene transfer in the tannery WWTP.

Recently, Li and coworkers (2015) compared the metagenomic libraries of total and plasmid DNA from influent, activated sludge and digested sludge of two WWTPs in Hong Kong. They observed that, compared to total DNA metagenomes, plasmid metagenomes encoded significantly higher numbers of ARGs. This emphasizes the prominent role of HGT in WWTPs. They also observed a considerable decrease in the number of ARGs in activated and digested sludge metagenomes compared to the influent metagenome.

Despite the many advantages of high-throughput shotgun-sequencing methodologies to identify the structure of biological wastewater treatment communities, as well as the diversity of WWTP resistomes, these approaches do not provide definitive relationships of ARGs to their host microorganisms. This may lead to varied correlations between WWTP resistome content and the corresponding microbiome (Noyes et al., 2016). In addition, the scope of metagenomic read mapping approaches is limited to prior knowledge of resistance genes (i.e. through comparison of the sequence reads to known ARGs). In this respect, function-based metagenomics are more valuable approaches as they have the potential to identify novel ARGs and MGEs and to correlate resistance genes with the community structure. A functional metagenomics approach involves construction of metagenomic libraries through extraction of DNA, cloning DNA fragments, heterologous expression in surrogate hosts and screening for specific activities (Lam et al., 2015).

Functional metagenomic studies of AR in environmental microbiomes, such as soil and marine water, have added considerably to our knowledge of the diversity of the natural gene pool of ARGs and revealed many unknown functions (Torres-Cortés et al., 2011; Donato et al., 2010; Schmieder and Edwards, 2012; Hatosy and Martiny, 2015). However, only limited studies have constructed functional metagenomic libraries from compartments of wastewater treatment processes and studied the diversity of ARGs and their host organisms (Amos et al., 2014; Uyaguari et al., 2011; Li et al., 2015). In a recent publication, Munck and coworkers (2015) combined metagenomic functional selections and deep metagenomic sequencing data to identify the diversity of ARGs in a core WWTP resistome in Denmark. They found that the core resistome consists of stably maintained and (mostly) novel ARGs which confer resistance to the 15 antimicrobials tested. They also showed that the WWTP microbial community is remarkably stable with a strong correlation between the resistome and the microbial composition and limited gene transfer with the human gut microbiota.

In recent years, integrated “omics” analyses have provided an enhanced understanding of the species present and their functions in wastewater microbial systems (Narayanasamy et al., 2015). Applying this approach, Roume and coworkers (2015) showed that seasonal variations did not dramatically affect the expression of enzymes involved in nitrogen (N) metabolism in the anoxic tank of a biological WWTP in Luxembourg. However, in winter when lipid accumulation was higher, they observed considerable expression of enzymes involved in glycerolipid metabolism. As an integrated “omics” analysis identifies the links between genes encoding key biological functionalities and functionally important community members, it can be used to optimize the wastewater treatment processes. This can be done through enrichment of favorable microorganisms such as lipid-accumulating organisms as proposed by Roume and coworkers (2015).

There is much current research focused on gaining a better understanding of the role of wastewater treatment in propagation and selection of AR. Current information suggests that WWTPs serve as a nexus between contaminants in human waste and the environment. However, there are still many gaps in our knowledge which need to be addressed to help understand whether WWTPs are a minor, major or variable contributor to the worldwide problem of AR.

Along with the necessity of understanding the origins of resistance and their functions, one of the potential approaches to the worldwide concern of AR is generating novel antimicrobials which have narrow spectra of action. Novel creative approaches can likely lead to the discovery of many new bioactive compounds in nature and as Strachan and Davies (2016) stated, “The entirely man-made global plague of resistance could/should have been prevented by stricter control of the use of antimicrobials. Without strict compliance and proper regulations, the evolution and dissemination of AR can never be prevented.”

1.3 Enhanced Biological Phosphorus Removal (EBPR) Process

In the present research, EBPR was selected as the wastewater treatment process configuration of interest to study AR. As a sustainable treatment technology capable of efficient simultaneous nitrogen (N) and phosphorus (P) removal performance, the EBPR variant of the activated sludge process has gained increasing attention in recent years (Oehmen et al., 2007; Seviour et al., 2003).

Phosphorus (P) is a key element of DNA, RNA and adenosine triphosphate (ATP) which plays an important role in energy metabolism, lipid transport and enzyme activation (Rieuwerts, 2015). While P has very limited natural reservoirs on earth and there is an ever-increasing demand for it for agricultural purposes, most of the P contained in municipal wastewater is wasted through treated effluent discharge to receiving waters or is disposed of in incinerators or landfills. Discharged P from WWTPs accelerates the eutrophication process in receiving water bodies and deteriorates the water quality. In response to the concerns regarding the continued pollution of water bodies with nutrients discharged from WWTPs, biological P removal from wastewater (without the need for chemical precipitants) is considered as an optimal approach.

Luxury uptake as the principal mechanism of biological P removal was first defined by Levin and Shapiro (1965). They showed that P is accumulated as intracellular polyphosphate (poly P) in polyphosphate-accumulating organisms (PAOs) and can be subsequently removed during biosolids wasting (Nielsen et al., 2010; Oehmen et al., 2007; Seviour et al., 2003).

In EBPR, P removal is achieved through recycling the biomass through alternating

anaerobic and aerobic conditions (Moretti et al., 2011; Seviour et al., 2003). In the anaerobic zone, PAOs take up carbon sources mainly in the form of short chain fatty acids (SCFAs) (e.g. acetate), from the influent and store them in the form of polyhydroxyalkanoates (PHAs) (Bahadoorsingh, 2010). The energy required for this biotransformation is mainly supplied by the hydrolysis of internally stored glycogen and poly P and the release of orthophosphate from the cell (Oehmen et al., 2007). In a subsequent aerobic zone, PAOs oxidize their internally stored PHAs as the carbon energy source for growth and glycogen is produced (Lawson, 2014).

The generation of reducing power required for the conversion of acetate, the primary substrate, to PHA, was explained by the Comeau-Wentzel model and the Mino model (Comeau et al., 1986; Wentzel et al., 1986; Mino et al., 1998). Based on the Comeau-Wentzel model, under anaerobic conditions, a part of the acetate is oxidized to carbon dioxide (CO_2) by the tricarboxylic acid (TCA) cycle for supplying the reducing power in the form of reduced nicotinamide adenine dinucleotide (NADH). In the Mino model, however, reducing power was assumed to be produced from anaerobic degradation of internally stored carbohydrates (glycogen) to pyruvate via the Embden–Meyerhoff–Parnas (EMP) pathway. More recent genomic data have suggested that reducing power likely comes from combining pathways of glycogen degradation and partial functioning of the TCA (Pereira et al., 1996; Burow et al., 2008).

Recent advances in molecular biology and high-throughput sequencing have revealed that despite the diverse EBPR ecosystems which are a function of the differences in wastewater composition, operation and configurations of WWTPs, EBPR systems share identical or closely related core bacterial communities (Nielsen et al., 2010; Oehmen et al., 2007). Key microbial players with functional relevance to EBPR systems include ammonia oxidizers, nitrite oxidizers, denitrifiers, PAOs, fermenters, hydrolyzers (proteins, polysaccharides and lipids) and filamentous organisms (Nogueira and Melo, 2006; Andreasen and Nielsen, 2000; Thomsen et al., 2007; Beer et al., 2006; He et al., 2007).

1.4 Membrane Bioreactors (MBRs)

To satisfy the ever-increasing requirement to produce high quality wastewater effluent for reclamation purposes, MBR technology has been proposed as an attractive solution (Zhang and Hall, 2006). MBRs eliminate the need for secondary clarifiers and tertiary filters in conventional activated sludge plants. Briefly, biological oxidation with coarse bubble aeration, as well as membrane physical separation, play the key roles in MBR treatment process.

It has been shown that MBRs could be used as an alternative solids-liquid separation technique to provide enhanced removal of pollutants such as residual organics, nutrients, suspended solids, microorganisms and inorganic metal ions (Barakat, 2011; Monti et al., 2007). Some studies have also proposed the application of MBR as an alternative technique to achieve more extensive antibiotic removal (Michael et al., 2013; Luo et al., 2014).

Coupling of MBR technology with the EBPR processes has been the focus of many studies in the past years (Yue, 2017; Winkler et al., 2016; Monti et al., 2007). Hall and coworkers (2010) showed that substitution of a secondary clarifier with membrane filtration leads to a shift in the composition of an EBPR bacterial community. Other research reported that MEBPR processes are capable of complete nitrification independent of system operating conditions (Monti et al., 2007).

The advantages of the MBR technology over secondary clarifiers include enhanced effluent quality, small footprint, reduced problems associated with sludge bulking and rising, potential to maintain high concentrations of suspended solids in mixed liquor, higher volumetric loading rates, low-rate sludge production and potential for operating systems at longer SRTs (Iorhemen et al., 2016; Monti et al., 2007).

Due to the potential to reduce excess sludge production and maximize the P concentration in mixed liquor, the operation of MEBPR systems at long SRTs has gained increasing attention in the past years (Li and Wu, 2014; Fenu et al., 2010; Britton et al., 2005; Xia et al., 2014). Operating at long SRT decreases the excess sludge production through enhanced endogenous respiration or bacterial endogenous decay rate (Li and Wu, 2014). The

feasibility of the operation of MEBPR processes at a 60 day SRT for P recovery was recently studied by Yue (2017). Comparing the levels of total P in parallel systems operating at 25 and 60 day SRTs, he observed that increasing SRT to 60 days almost doubled the concentration of total P in the aerobic mixed liquor.

Utilizing membrane filtration for solids-liquid separation may also cause some operational challenges. The most commonly reported problems associated with the application of MBR technology include membrane fouling caused by the existence of extracellular polymeric substances (EPSs), DO poisoning of the anoxic (or aerobic) zone, increased operational energy costs, high costs of membrane replacement due to integrity issues and foaming (Di Bella et al., 2011; Yue, 2017).

Contrary to conventional activated sludge WWTPs, where foam formation is mostly due to the overgrowth of microorganisms which produce biosurfactants and/or have a high cell hydrophobicity (e.g. *Gordonia amarae*), studies have suggested EPSs as the major cause of biological foaming of mixed liquor in MBRs (Nakajima and Mishima, 2005; Cosenza et al., 2013). EPSs are high molecular weight natural polymers which are released by microorganisms under stressed conditions to form biofilms (Mathur et al., 2015). Di Bella et al. (2011) showed that retention of EPSs in MBR plants is higher than that of conventional wastewater treatment systems. As foam formation interferes with the estimation of total suspended solids (TSS) in the entire system, regular harvesting of foam bacteria in MEBPR systems has been proposed as an efficient strategy to reduce the accumulation of foam and control SRT (Yue, 2017; Hall et al., 2011).

As long as membranes are intact and there are no defects in the integrity of the membrane surface, MBRs can achieve the complete removal of microorganisms and a vast majority of viruses. However, anomalies with the membrane surface and/or the filtration system leads to microbial and viral penetration of the product water. Hai and coworkers (2014) summarized the causes of breaches in the membrane system to a few major classes including pore expansion due to exposure to chemical agents during cleaning; chemical degradation by disinfection oxidants; and mechanical damage due to high local shear forces and vibrations. Robinson and colleagues (2015) reviewed the changes in membrane properties, such as membrane physical and chemical characteristics, as well as performance factors, as a result

of the long-term use of membranes (membrane aging). They also showed that performance factors, such as breach frequency, are strongly linked to chemical and physical characteristics of the membrane including the pore porosity and geometry as well as polymer phase and molecular weight. Monitoring the membrane integrity is usually performed through the application of on line and off line testing methods. While standard methods for online testing include marker-based tests (i.e. particle passage), particle counting, turbidity and biological monitoring, off line testing methods such as pressure-based methods (e.g. air pressure tests, vaccum holding tests) have also been proposed (Lousada-Ferreira et al., 2016; Guo et al., 2010).

1.5 Research Objectives on the Role of MEBPR Processes in the Spread of Antimicrobial Resistance

Although conventional wastewater treatment systems have been largely studied with respect to the evolution, spread and positive selection of ARB and ARGs (Auerbach et al., 2007; Zhang et al., 2009; Reinthaler et al., 2003; Gallert et al., 2005; Schwartz et al., 2003; Ramsden et al., 2010; Yang et al., 2012; Galvin et al., 2010; Ma et al., 2011), the possible roles of advanced treatment technologies, such as EBPR processes, in the distribution of AR has been less studied. Hence, the present project is specifically focused on understanding the role of a MEBPR process in the release of ARB and ARGs.

A summary of the current research gaps in the role of wastewater treatment in the distribution of ARB and ARGs was provided in Section 1.2. With respect to the role of MEBPR processes, the following topics need to be addressed:

- Many researchers in the past years have studied the occurrence of ARB and ARGs in raw influent, treated effluent and the environmental ecosystems affected by the by-products of wastewater treatment systems, including effluent discharged to receiving waters and wastewater irrigated soil (Broszat et al., 2014; Sigala and Unc., 2012; Szczepanowski et al., 2009; Reinthaler et al., 2003; Lefkowitz and Duran., 2009; Luczkiewicz et al., 2010; Jury et al., 2011; Huang et al., 2012). In most of these studies, the performance of WWTPs has only been evaluated through comparing the

ARB proportions and/or ARGs levels between influent and treated effluent. Hence, the efficiency of the solids-liquid separation techniques in the attenuation or promotion of AR restricted our view of the role of wastewater treatment processes. To satisfy this gap, antimicrobial susceptibility profiles and the behaviour of ARB as well as the variation in ARG concentrations as they travel through the redox zones of MEBPR systems need to be explored.

- As an important parameter which enhances our understanding of the fate of ARB in advanced wastewater treatment processes, the significance of the role of system operating conditions such as SRT requires further investigation. Current efforts in the field of wastewater engineering to minimize excess sludge production through operating systems at long SRTs need to be coupled with AR studies, so that the environmental risks associated with the discharge of ARB in waste aerobic mixed liquor can be evaluated. To address the knowledge gap regarding the role of long SRT operations in dissemination of AR, performance assessment needs to be done through comparison of the ARB percentage or ARG levels between the control (normal range of SRT between 15 to 40 days) and long SRT MEBPR systems which are operating under identical influent input and strict parallel conditions.
- Foam formation in MBRs can cause diverse operational challenges including interference with TSS measurements and the accurate estimation of SRT, production of foul odors in warm climates and the potential of the reactor freeboard overflowing (Di Bella et al., 2011). The constant formation of foam in MEBPR processes was first reported by Monti et al. (2006) who observed a greater accumulation of foam on the surface of the anoxic reactor in a MEBPR train compared to that of a conventional EBPR system. Hall et al. (2011) proposed regular harvesting of foam bacteria in MEBPR processes as a means to control SRT.

  Characterizing foam as an alternative resource for sidestream P recovery, Hall et al. (2011) compared the composition of microbial communities as well as the levels of total Kjeldahl nitrogen (TKN), total P concentrations and volatile suspended solids (VSS)/TSS ratios between foam and underlying mixed liquor. While they observed about 80% similarity in the bacterial communities of foam and anoxic mixed liquor,

chemical analysis revealed similar contents of P, N and organic matter in the two environments. In a pilot-scale comparative study of MEBPR systems at different SRTs, Yue (2017) observed that foam generation increased considerably by increasing SRT from 25 to 60 days and foam was the major route to waste solids from the 60 day SRT train. MEBPR foam, wasted only for the purpose of controlling SRT or applied as an alternative resource for P recovery, requires comprehensive studies with respect to AR.

To our knowledge, no studies have attempted to compare the patterns of AR between foam and waste aerobic mixed liquor heterotrophic bacteria in MEBPR processes. To gain insight on the causes of foam formation in MEBPR processes, the taxonomic composition of foam and underlying mixed liquor bacterial communities need to be identified and compared.

- While sequence-based metagenomics can improve our knowledge of the diversity of ARGs in WWTP environments, the scope of these techniques is restricted to identification of well-characterized genes. In this respect, functional metagenomic approaches could be used to investigate the diversity of the natural gene pool of ARGs with unknown functions. So far, only limited studies have attempted to study the diversity of ARGs in wastewater using a functional metagenomics approach (Amos et al., 2014; Uyaguari et al., 2011; Li et al., 2015; Munck et al., 2015). Considering foam as a by-product of MEBPR processes, further studies are required to identify the diversity of ARGs in this microbiome using polymerase chain reaction (PCR)-based and functional metagenomic techniques.
- Another knowledge gap is that most studies that have focused on resistance patterns of ARB in WWTPs have only focused on studying limited subsets of culturable pathogenic bacteria and very little is known about AR levels in total cultivable heterotrophic bacteria (i.e. bacteria that use organic compounds as a source of carbon and energy) in advanced wastewater treatment systems. Due to the high correlation between indicators of faecal contamination and pathogenicity, faecal coliforms, *E. coli* and *Enterococci spp.* have been frequently studied in WWTPs (Figueira et al., 2011; Nagulapally et al., 2009; Luczkiewicz et al., 2010; Iversen et

al., 2002; West et al., 2011; Holzel et al., 2010). Some research has also focused on resistance populations of opportunistic pathogens such as *Aeromonas* and *Pseudomonas* species in wastewater (Gallert et al., 2005; Al-Bahry et al., 2009).

Studying the ARB patterns of total cultivable heterotrophic bacteria in MEBPR systems is necessary as it provides an opportunity for monitoring a broader range of antibiotic resistant human pathogens such as the ESKAPE (*Enterococcus faecium, Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) pathogens. As the leading cause of nosocomial infections, the ESKAPE group includes Gram-positive (G+) and Gram-negative (G-) pathogens that escape the effects of antimicrobials due to multiple drug resistance (Rice, 2008; Behroozian et al., 2016). Except for *Enterococcus faecium* (which is an indicator of human fecal contamination), ESKAPE pathogens such as *Staphylococcus aureus*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* are not of fecal origin.

Knowing that the majority of wastewater bacteria cannot be cultivated under laboratory conditions (Wagner et al., 1993), determination of the percentage of ARB in total cultivable heterotrophic bacteria, which include both pathogenic and nonpathogenic commensal bacterial communities, provides a more accurate assessment of AR levels in MEBPR systems compared to indicator organisms.

Previous studies have shown that the composition of the media, incubation time and temperature are variables that have direct impacts on the diversity of bacteria growing on a medium (Gensberger et al., 2015; Vieira and Nahas, 2005). In this respect, monitoring the resistance patterns of culturable heterotrophic bacteria using two non-selective non-differential media (high nutrient (e.g. MH) vs low nutrient (e.g. R2A)) could provide an enhanced understanding on the role of the media composition (influencing the diversity of tested microorganisms) on the evaluation of wastewater treatment performance in attenuation or amplification of AR.

- While studies indicate the efficiency of MBR technology in the removal of antibiotics, ARB and ARGs (Munir et al., 2011; Xia et al., 2012), the occurrence of indicator

organisms in membrane permeates is well documented (Jacangelo et al., 1991; Hai et al., 2014). Recently, Harb and Hong (2017) studied the abundance and log removal of some pathogenic species in the membrane-treated effluent of a full-scale MBR plant as well as a lab-scale anaerobic MBR. In both MBR effluents, they observed opportunistic pathogens.

Membrane-treated effluent is not often reused or exposed to direct human contact without supplemental disinfection processes such as ultraviolet radiation (UV), chlorination or ozonation. While it has been shown that disinfection of WWTP effluent can cause substantial reduction in the abundance of ARB and ARGs, some studies have reported that post-treatment processes such as ozonation and chlorination can select for opportunistic bacteria and associated ARGs in treated effluent (Alexander et al., 2016; Hu et al., 2016; Huang et al., 2011; Zhang et al., 2015a).

Membrane integrity is not routinely monitored by indirect biological monitoring tests such as bacterial/viral counts. Hence, knowledge of the bacterial diversity and resistance patterns of ARB in MBR effluents is still limited. To satisfy this knowledge gap and to compensate for the drawback of using indicator bacteria, further studies are required to monitor AR patterns of total heterotrophic bacteria in membrane-treated effluent in MEBPR processes.

The present project was intended to address the following research questions.

1. Do long SRT operations elevate the concentrations of the *sul*1 gene (normalized to 16S rRNA) and the proportions of ARB in total cultivable heterotrophic bacteria in mixed liquor and treated effluent of MEBPR processes, compared to those of the influent?
2. How do prolonged SRT operations influence the release rates of ARB in waste aerobic mixed liquor, foam and membrane-treated effluent in MEBPR systems?
3. How efficient is the MEBPR process in removal of total and resistant cultivable heterotrophic bacteria from influent?
4. How diverse are the cultivable ARB in membrane-treated effluent of a MEBPR

system?

5. How can the taxonomic composition of bacterial communities in influent, mixed liquor, foam and membrane-treated effluent be compared in a model MEBPR process?
6. What is the diversity of ARGs in the MEBPR foam?

1.6 Research Approach

In order to address the research questions described in Section 1.5, the present study applied both culture-dependent and molecular techniques to gain knowledge of the possible role of MEBPR processes in the distribution of AR. Three major aspects of MEBPR processes which were studied in this project included: (1) the role of long SRT operations in ARB and ARG release in waste aerobic mixed liquor, foam and membrane-treated effluent, (2) diversity and resistance profiles of membrane-treated effluent heterotrophic bacteria and, (3) diversity of ARGs and the taxonomic composition of bacterial communities in waste foam.

In order to provide an improved understanding of the environmental risks associated with the release of ARB and ARGs from MEBPR processes operating at long SRTs, the performance of the University of British Columbia (UBC) pilot plant MEBPR systems operating at 25 (as a control) and 60 days of SRT were monitored. It should be noted that prior to sampling from the 60 day SRT train for the purpose of the current project, the feasibility of the operation of the MBPR system at SRT = 60 days was studied and comparable phosphorus (P) and organic carbon removal in MEBPR systems operating at 25 and 60 day SRTs were reported (Yue, 2017).

Chapter 2 provides a summary of materials and methods used to examine the objectives of this study. Each method cited in this chapter has been applied one or more times during the progression of the project. As an example, in all three subsections of the “Results and Discussion” chapter, the methodologies for DNA extraction and performing PCR reactions (Sections 2.4.2.1 and 2.4.2.2) were applied.

Chapter 3, “Results and Discussion”, presents the observations of three separate projects each of which focused on addressing one or two research questions. Section 3.1, “Release

of SMX^r Bacteria from Membrane Effluent and Levels of the *sul*1 Gene in MEBPR Processes", has two subsections. In the first subsection, sulfamethoxazole-resistant (SMX^r) cultivable heterotrophic bacteria isolated from influent and treated effluent of the UBC MEBPR pilot plant operating at a 25 day SRT were studied in terms of bacterial log removal, antimicrobial susceptibility profiles as well as bacterial diversity. In detail, the efficiency of the MEBPR system was evaluated by comparing the colony counts of total heterotrophic and/or SMX^r bacteria between influent and membrane-treated effluent. To examine if the percentage of ARB in SMX^r bacteria changed through the MEBPR process, antimicrobial susceptibility profiles of a total of 120 influent and MEBPR effluent SMX^r bacterial isolates (against a set of five antibiotics) were compared between influent and membrane effluent. For the 120 SMX^r strains tested, 16S rRNA gene sequencing was performed for colonies showing at least one resistance to another class of antibiotic and the diversity of SMX^r bacteria in influent and membrane-treated effluent was identified at the genus level. In the second subsection, the role of very long SRT operations (25 vs 60 days) on the concentrations of a SMX resistance gene (*sul*1 gene) in the dual trains of the UBC pilot plant was studied. In order to evaluate the performance of membrane filtration in removal of the *sul*1 gene, gene copies normalized to the 16S rRNA gene copies (relative concentration), as well as sample volume (absolute concentration) were compared between influent and effluent.

Section 3.2 named "The Role of Long SRT Operations in Release of ARB from MEBPR Processes" includes five separate subsections. In the first part, the patterns of AR in total cultivable heterotrophic bacteria isolated from influent, anoxic mixed liquor (as representative of redox reactors), foam as well as treated effluent in the parallel UBC pilot plant treatment facilities operating at 25 and 60 day SRTs were compared. To provide performance comparison, dual trains were operated under identical system operating conditions, with the exception of SRT variations. To ensure broad representation of environmental bacteria, > 7,000 isolated bacterial colonies were screened for resistance to five antimicrobials (sulfamethoxazole (SMX), trimethoprim (TMP), tetracycline (TET), ciprofloxacin (CIP), amoxicillin (AMX)) from five different classes in liquid cultures. As previously noted in Section 1.5 and to statisfy the knowledge gap regarding the role of media

composition in wastewater treatment performance assessment, screening was done using a nutrient-high (Müeller-Hinton (MH) and a nutrient-low (Reasoner's 2A (R2A) medium.

In the second subsection, the total number of resistances in each of the tested isolated bacteria was calculated so that the MDR patterns of heterotrophic bacteria in influent and parallel MEBPR processes could be compared. For each of the five tested antimicrobials, percent removal of ARB was determined in parallel bench-scale wastewater treatment systems and data are reported in the third subsection. In the fourth subsection, the role of SRT variations on the release rates of ARB through disposal of excess aerobic mixed liquor, foam, as well as discharge of MEBPR treated effluent was studied. In this subsection, the contributions of waste aerobic mixed liquor, foam and membrane-treated effluent in release of ARB were compared and major routes of ARB discharge from MEBPR processes were assessed. It was assumed that differences in patterns of resistance observed in MH and R2A heterotrophic bacteria were due to the differences in the culturable community composition of testing media. To test this hypothesis, 192 random effluent bacteria (96 MH bacteria and 96 R2A bacteria) were characterized based on their lactose fermentation patterns by growth on MacConkey plates. Data are presented in the fifth subsection. To study the diversity of cultivable heterotrophic ARB in membrane-treated effluent, 33 of the most different-looking colonies (in terms of pigmentation, size and shape) which exhibited diverse AR profiles against five tested antimicrobials were then identified by sequencing the taxonomic marker gene (16S rRNA).

Section 3.3 named "Assessment of Bacterial Communities in a Model MEBPR System and Identification of ARGs in the Foam Microbiome" is divided into two separate subsections. In the first part, the taxonomic composition of bacterial communities in a MEBPR process at the control SRT (25 days) was profiled using high-throughput sequencing of the 16S rRNA gene V4 amplicons. The table of operational taxonomic units (OTU) was obtained from clustering the quality-filtered generated Fastq files into 97% similarity OTUs using the Mothur software. Bubble plots of order level abundance as well as hierarchal dendrograms corresponding to abundant (relative abundance > 1%), intermediate (relative abundance between 1% and 0.1%) and rare taxa (relative abundance < 0.1%) for all four samples of influent, mixed liquor, foam and membrane-treated effluent were prepared using the R

packages. Findings from this subsection were not only used to compare the relative abundance of bacterial communities between foam and underlying mixed liquor, but also helped in tracking the shift in the bacterial communities from influent to EBPR mixed liquor and finally to treated effluent. In the second subsection, the diversity of ARGs in MEBPR foam was investigated using functional metagenomics and PCR-based approaches. Functional screening of metagenomic libraries to isolate novel ARGs is a relatively recent topic in the field of wastewater engineering. To my best knowledge, no studies have attempted to search for the diversity of ARGs and their host organisms in any of the compartments of MEBPR processes, including that of foam bacteria. Observations from this subsection may contribute basic knowledge for the occurrence of low frequency genes in foam generated in MEBPR systems.

2 MATERIALS AND METHODS

2.1 Test Facility

The present research was conducted at the UBC wastewater treatment pilot plant over the period September 2013 to August 2016. The pilot plant is a dual-train facility, with identical reactor trains configured for the typical University of Cape Town (UCT) membrane enhanced biological phosphorus removal (MEBPR) process. Each train consists of one compartmentalized bioreactor in which a sequence of an anaerobic zone, an anoxic zone and an aerobic zone is employed. During the study period, parallel trains were operating under steady-state conditions at 25 and 60 day SRTs. **Figure 2.1** represents the configuration of the dual-train processes utilized in the present comparative study.

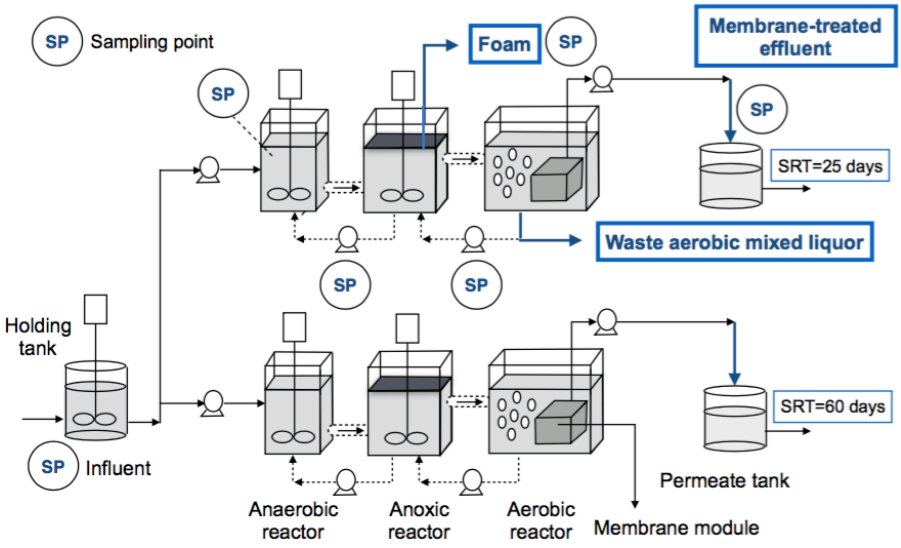


Figure 2.1 Schematic of the parallel MEBPR systems with sampling points and locations

The feed to the dual-train facility was municipal sewage which was pumped from a sewer, collecting wastewater from residential areas on the UBC campus, to three large equalization tanks. The wastewater in the equalization tanks was pumped continuously to a primary

clarifier while the clarifier effluent flowed by gravity to a small holding tank. Clarifier discharge ("influent" in **Figure 2.1**) was pumped to each anaerobic reactor using a separate pump. Activated sludge in the anaerobic reactors flowed by gravity to the anoxic and then aerobic tanks and was finally membrane-filtered and then pumped to the permeate tank.

In the MEBPR trains, influent was mixed with activated sludge from the anoxic recycle line in the anaerobic zone. The addition of acetate in the anaerobic zone together with return biomass from the anoxic zone stimulated the growth of polyphosphate-accumulating organisms (PAOs) and biological phosphorus (P) release. In the anoxic zone where denitrification occurs, activated sludge was mixed with biomass from the aerobic recycle line. Organic carbon oxidation, nitrification and P uptake occurred in the aerobic zone where adequate air flow was supplied by coarse bubble aeration.

Separation of solids and liquid in the UBC pilot plant MEBPR processes was done by ZeeWeed custom-built polyvinylidene fluoride hollow fiber modules which were directly immersed in the aerobic reactors. The nominal pore size of the membrane modules was 0.04 μm and each module had a surface area of 12 m^2. Membranes were operated under a constant flux of 12 L/m^2h in two modes of permeation (9.5 minutes) and rapid backflush (30 seconds).

Daily operations of the UBC pilot plant included temperature and dissolved oxygen monitoring in the aerobic zones, testing of the trans-membrane pressure to determine if chemical cleaning is needed, biomass solids wasting for controlling SRT and finally, collecting samples for metals and inorganic chemical analysis for monitoring the process performance (Bahadoorsingh, 2010). **Table 2.1** provides a summary of the performance data in UBC MEBPR systems (over the period of December 2014 to February 2015). UBC pilot plant general operating conditions are also provided in **Table 2.2**.

Table 2.1 UBC pilot plant performance data

(monitored period: December 2014 to February 2015)

Parameter	Influent	Parameter	Membrane effluent (SRT = 25 days)	Membrane effluent (SRT = 60 days)
TSS (mg/L) (n=10)	112 (σ=31)	TSS (mg/L)	-	-
VSS (mg/L) (n=10)	92 (σ=36)	VSS (mg/L)	-	-
COD (mg/L) (n=52)	289 (σ=80.5)	COD (mg/L) (n=52)	27 (σ=12.5)	29 (σ=11)
Total P (mg/L) (n=13)	4.5 (σ=0.6)	Total P (mg/L) (n=13)	0.4 (σ=0.17)	0.45 (σ=0.17)
P_{in} (g/day) (n=13)	23 (σ=2.6)	P_{out} (g/day) (n=13)	2 (σ=0.8)	2.3 (σ=0.7)
PO_4-P (mg/L) (n=60)	2.2 (σ=0.5)	PO_4-P (mg/L) (n=60)	0.01 (σ=0.02)	0.04 (σ=0.03)
TKN (mg N/L) (n=13)	38.7 (σ=4.5)	TKN (mg N/L) (n=13)	1.14 (σ=0.5)	0.9 (σ=0.3)
NH_4-N (mg/L) (n=60)	36.9 (σ=5.5)	NH_4-N (mg/L) (n=60)	0.15 (σ=0.4)	0.04 (σ=0.05)
NO_3-N (mg/L) (n=60)	0.01 (σ=0.02)	NO_3-N (mg/L) (n=60)	14.2 (σ=2.3)	9.8 (σ=2.5)
Total N (mg/L) (n=13)	37.6 (σ=3.5)	Total N (mg/L) (n=13)	15.3 (σ=2.4)	10.1 (σ=2.6)
Parameter	**Anaerobic mixed liquor (SRT = 25 days)**	**Anoxic mixed liquor (SRT = 25 days)**	**Aerobic mixed liquor (SRT = 25 days)**	**Foam (SRT = 25 days)**
Total P (mg/L)	90.4 (σ=26.7)	145.9 (σ=14.2)	288.6 (σ=30.4)	1132.9 mg/kg (σ=183.8)
TKN (mg N/L)	188.9 (σ=16.9)	323.8 (σ=19.6)	624.5 (σ=38.5)	2380.6 mg/kg (σ=245.4)
PO4-P (mg/L)	14.0 (σ=2.2)	4.1 (σ=1.6)	0.13 (σ=0.06)	-
NH_4-N (mg/L)	27.8 (σ=4.9)	17.9 (σ=3.6)	1.2 (σ=4.7)	-
NO_3-N (mg/L)	0.1 (σ=0.1)	0.47 (σ=0.5)	14.6 (σ=2.3)	-
TSS (mg/L)	2331 (σ=385)	4160 (σ=282)	7912 (σ=435)	33 g/kg (σ=1.3)
VSS (mg/L)	2113 (σ=261)	3577 (σ=252)	6604 (σ=363)	29 g/kg (σ=1.3)
Parameter	**Anaerobic mixed liquor (SRT = 60 days)**	**Anoxic mixed liquor (SRT = 60 days)**	**Aerobic mixed liquor (SRT = 60 days)**	**Foam (SRT = 60 days)**
Total P (mg/L)	209.5 (σ=49.1)	463.2 (σ=19.1)	683.4 (σ=38.1)	1413.7 mg/kg (σ=207.7)
TKN (mg N/L)	389.2 (σ=91)	835.54 (σ=36.5)	1203.7 (σ=74.9)	2661.6 mg/kg (σ=327)

Parameter	Anaerobic mixed liquor (SRT = 60 days)	Anoxic mixed liquor (SRT = 60 days)	Aerobic mixed liquor (SRT = 60 days)	Foam (SRT = 60 days)
PO4-P (mg/L)	22.9 (σ=3.39)	4.7 (σ=5.2)	0.2 (σ=0.1)	-
NH_4-N (mg/L)	25.9 (σ=4.5)	10.4 (σ=2.6)	1.2 (σ=3.6)	-
NO_3-N (mg/L)	0.07 (σ=0.09)	0.16 (σ=0.2)	8.8 (σ=2.9)	-
TSS (mg/L)	4679 (σ=1166)	10840 (σ=548.22)	15648 (σ=634)	38 g/kg (σ=1.1)
VSS (mg/L)	3804 (σ=743)	8174 (σ=2319.01)	12638 (σ=467)	33 g/kg (σ=1.0)

-: not measured, n: number of sampling events

Table 2.2 UBC pilot plant operating conditions

(monitored period: December 2014 to February 2015)

Parameter	Value	Parameter	Value
Aerobic recycle ratio	1:1	Anoxic recycle ratio	1:1
Anaerobic zone volume (L)	245	System hydraulic retention time (HRT) (hr)	10
Anoxic zone volume (L)	624	Nominal aerobic dissolved oxygen concentration (mg/L)	2.5-3
Aerobic zone volume (L)	1359	Acetate feed rate (g/L @ 10 mL/min)	14
Total volume of compartmentalized bioreactor (L)	2228	Influent daily flow rate (L/min)	3.7
Foam wastage rate (kg/day) (SRT = 25 days) (n=63)	5.8 (σ=1.9)	Foam wastage rate (kg/day) (SRT = 60 days) (n=63)	13.2 (σ=0.7)
Aerobic mixed liquor wastage rate (L/day) (SRT = 25 days) (n=63)	43.5 (σ=8.7)	Aerobic mixed liquor wastage rate (L/day) (SRT = 60 days) (n=63)	0.2 (σ=1.2)

2.2 Sampling Plan

As mentioned in Section 1.6, observations from the present project are provided under three separate subsections (3.1, 3.2 and 3.3), each addressing one or two research questions of the study. In each section, depending on the objectives to be satisfied, the sampling plans including the points and dates of sample collection, were different and a summary is provided in **Table 2.3**. As observed, the sampling plan was presented based on the

particular methodologies applied to address the project objectives in each section. Briefly, in Section 3.1, quantitative polymerase chain reaction (qPCR) and monitoring of bacterial growth on solid media were the main methodologies used. In Section 3.2, total cultivable heterotrophic bacterial isolates were screened for AR by growth in liquid media. To address the objectives of the project in Section 3.3, high-throughput sequencing, as well as construction and screening of the foam metagenomic library, were performed. As observed in **Table 2.3**, in several cases, the same samples (biological replicates) collected from one tank at a specific time were the subject of discussion in more than one section.

Table 2.3 Summary of the sampling schedule including dates and locations

Sample	SRT (days)	January 7^{tn}, 2014	June 16^{tn}, 2014	August 7^{tn}, 2014	December 3rd, 2014	January 6^{tn}, 2015	February 3rd, 2015
Influent	-	3.1-1 3.1-2	3.1-2	3.3-2	3.1-2,3.2,3.3-1	3.1-2,3.2, 3.3-1	3.1-2,3.2, 3.3-1
Anaerobic mixed liquor	25	-	3.1-2	3.3-2	3.3-1	3.1-2,3.3-1	3.1-2, 3.3-1
Anoxic mixed liquor		-	3.1-2	3.3-2	3.1-2,3.2, 3.3-1	3.2, 3.3-1	3.1-2,3.2, 3.3-1
Aerobic mixed liquor		-	-	3.3-2	3.1-2,3.3-1	3.1-2,3.3-1	3.1-2,3.3-1
Membrane treated effluent		3.1-1 3.1-2	3.1-2	-	3.1-2,3.2, 3.3-1	3.1-2,3.2, 3.3-1	3.1-2,3.2, 3.3-1
Foam		-	-	3.3-2	3.2, 3.3-1	3.2, 3.3-1	3.2, 3.3-1
Anaerobic mixed liquor	60	-	3.1-2	-	3.1-2	3.1-2	3.1-2
Anoxic mixed liquor		-	-	-	3.1-2, 3.2	3.1-2, 3.2	3.1-2, 3.2
Aerobic mixed liquor		-	3.1-2	-	3.1-2	3.1-2	3.1-2
Membrane treated effluent		-	3.1-2	-	3.1-2, 3.2	3.1-2, 3.2	3.1-2, 3.2
Foam		-	-	-	3.2	3.2	3.2

3.1-1: ARB growth on solid media; 3.1-2: qPCR; 3.2: ARB growth in liquid media; 3.3-1: Illumina high-throughput sequencing; 3.3-2: Construction and screening of the functional metagenomic library

During sampling, three biological replicate samples of influent and mixed liquor taken at 10

to 15 minute intervals were collected from each point, with the exception of foam. All the foam formed on the surface of an anoxic tank over a period of 24 hours was collected in a 20 L plastic bucket, mixed well for five minutes and triplicate samples were taken. Grab samples of the reactor influent and anaerobic tanks were taken using 50 mL sterilized syringes about 40 centimeters below the surface layer, while samples from the anoxic and aerobic reactors were grabbed from the anoxic and aerobic recycle lines, respectively (**Figure 2.1**). After each biological replicate sample was well mixed, sterilized containers were used to store samples for each specific purpose. All samples were taken before wasting of aerobic mixed liquor and foam from the reactors on the sampling day. Depending on the future usage of the samples, sample handling and preservation procedures were different and a summary is provided in **Table 2.4**. An extra set of 15 mL sterilized falcon tubes was used as a backup to store triplicate samples of influent, redox reactors and foam samples at -80°C.

Table 2.4 Sample handling and preservation procedures

Purpose	Sample	Containers	Preservation and Storage
Bacterial plating	Influent, mixed liquor, foam	15 mL sterilized falcon tubes	Transfer on ice and immediate storage at 4°C, serial dilution and plating within six hours of sampling collection
	Membrane-treated effluent	2 L sterilized plastic bottles	Immediate storage at 4°C, vacuum filtration and plating within six hours of sampling collection
Genomic DNA extraction	Influent, mixed liquor, foam	50 mL sterilized centrifuge tubes	Centrifugation and flash freezing the pellet by liquid N on site, storage at -80°C
	Membrane-treated effluent	20 L acid-washed plastic carboys	Biomass concentration on 0.22 µm Sterivex filters within four hours of sampling, addition of 180 µL of sucrose lysis buffer (SLB), storage at -80°C

2.3 Target Antimicrobials

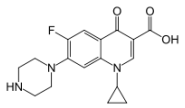
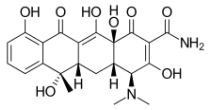
Depending on the objectives of the project, resistance to different and sometimes similar antimicrobials were studied in Sections 3.1, 3.2 and 3.3. In Section 3.1, the percentage of sulfamethoxazole-resistant (SMX^r) heterotrophic bacteria in influent and membrane-treated

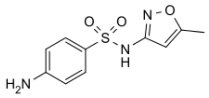
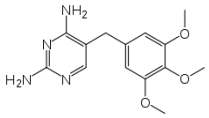
effluent were compared. In the same section and to determine if the proportions of ARB decreased from influent to treated effluent, the percentages of resistance to tetracycline (TET), trimethoprim (TMP), ciprofloxazin (CIP), amoxicillin/clavulanic acid (AMC), chloramphenicol (CM),) and kanamycin (KAN) were compared between influent and membrane effluent SMX^r bacterial communities.

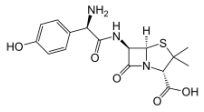
In Section 3.2, patterns of resistance to five antimicrobials (sulfamethoxazole (SMX, 50 µg/mL), tetracycline (TET, 10 µg/mL), amoxicillin (AMX, 32 µg/mL), trimethoprim (TMP, 5 µg/mL) and ciprofloxacin (CIP, 5 µg/mL)) were studied in the total heterotrophic bacteria isolated from influent, mixed liquor, foam and treated effluent to determine the effects of extended SRT operations on ARB release from the parallel MEBPR processes. **Table 2.5** summarizes some general properties of the tested antimicrobials in Section 3.2 and their corresponding resistance mechanisms. Five tested antimicrobials were broad spectrum, effective on a wide diversity of Gram positive (G(+)) and Gram negative (G(-)) bacteria, and were chosen to be representative of a particular antimicrobial class. **Table 2.5** shows that these antimicrobials have different modes of action and hence, different bacterial resistance mechanisms. As an example, resistance to ciprofloxacin as an inhibitor of DNA replication could be due to mutation in the target protein (e.g. *gyr*A gene) while resistance to tetracycline which is a protein synthesis inhibitor, could be efflux-mediated (e.g. *tet*(A) gene).

Due to their widespread usage, broad spectrum and persistence in aquatic environments, the fates of most of the target antimicrobials in Sections 3.1 and 3.2 including SMX, TET, TMP, AMX, AMC, CFM and AMP have been frequently studied in WWTP environments (Batt et al., 2007; Jia et al., 2012; Xu et al., 2007a) and resistance profiles of indicator bacteria against these antimicrobials have been well documented in WWTPs (Zhang and Zhang, 2011; Lefkowitz and Duran, 2009; Akiyama and Savin, 2010; Szczepanowski et al., 2009). Hence, selection of these antimicrobials provided a means for comparison of the observations of the present project with current knowledge.

Table 2.5 Classification, clinical usage, mechanisms of AR and mobility of ARGs in tested antimicrobials in the present research

Antimicrobial	**Ciprofloxacin (Fàbrega et al., 2009; Jung et al., 2009)** 
Bacterial host range	Wide range of G(-) and G(+) pathogens
Classification	Second generation quinolones/ synthetic/ bactericidal
Clinical usage	Treatment of lower respiratory tract infections, skin and soft tissue infections, sexually transmitted diseases and urinary tract infections
Mode of action	Inhibition of DNA replication by targeting DNA gyrase and topoisomerase IV enzymes. DNA gyrase comprises two subunits (A and B) which are encoded by the *gyr*A and *gyr*B genes, respectively. Topoisomerase IV comprises two subunits (A and B) which are encoded by the *Par*C and *Par*E genes, respectively
Mechanisms of resistance (encoded enzymes)	(1) Chromosomal mutation in genes encoding the protein targets (i.e. *gyr*A, *par*C genes) (2) Reducing the drug accumulation by: • Decreased uptake (e.g. mutations in the regulatory genes of the outer membrane porin proteins such as *omp*A in *E. coli*) • Increased efflux (e.g. efflux pump in the resistance/nodulation/division superfamily in which AcrAB proteins are overexpressed) (3) Presence of plasmid-located genes for target protection (e.g. *qnr*A, *qnr*B) (4) Acetylation (antibiotic inactivation by a modified acetyltransferase enzyme (encoded by the *aac*(6')-*Ib-cr* gene))
Mobility of ARGs	Plasmid-mediated *qnr* genes, *aac*(6')-*Ib-cr* gene and *qep*A gene
Antimicrobial	**Tetracycline (Chopra and Roberts, 2001)** 
Bacterial host range	Broad spectrum, effective against a wide range of G(+) and G(-) bacteria and atypical microorganisms such as rickettsiae
Classification	First generation tetracyclines/ naturally occurring molecule (from *Streptomyces aureofaciens*)/ bacteriostatic
Clinical usage	Treatment of skin, respiratory tract and urinary tract infections, and treatment of severe acne and sexually transmitted diseases such as chlamydia
Mode of action	Inhibition of protein synthesis by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor (A) site

Mechanisms of resistance (encoded enzymes)	(1) Efflux proteins (in the major facilitator superfamily (e.g. *tet(*A), *tet(*B), *tet(*C)) (2) Ribosomal protection proteins (e.g. *tet(*M), *tet(*O)) (3) Enzymatic inactivation of tetracycline (e.g. monooxygenation (*tet(*X))
Mobility of ARGs	The occurrence of: • G(-) Group 1 (e.g. *tet(*A), *tet(*B)) efflux genes on large plasmids (not mobile or conjugative) • G(+) Group 2 (e.g. *tet(*K), *tet(*L)) efflux genes on small transmissible plasmids integrated into chromosome or larger plasmids • Some ribosomal protection genes *(e.g. tet(*M)) on large conjugative transposons
Antimicrobials	**Sulfamethoxazole**  **Trimethoprim**  **(Eliopoulos and Huovinen, 2001; Podnecky et al., 2017)**
Bacterial host range	Broad sprectrum iof activity against G(-) (e.g. *E. coli* and other members of the family *Enterobacteriaceae*) and G(+) bacteria (e.g. *Streptococcus pneumoniae* and *Staphylococcus aureus*)
Classification (sulfamethoxazole)	Sulfonamides/ synthetic/ bacteriostatic
Classification (trimethoprim)	Pyrimidine/ synthetic/ bacteriostatic
Clinical usage	Treatment of unirary and respiratory tract infections and skin pathogens
Mode of action (sulfamethoxazole)	Blocking folate biosynthesis by inhibiting the dihydropteroate synthase (DHPS) enzyme which catalyzes the formation of dihydropteroic acid from para-aminobenzoic acid (PABA)
Mode of action (trimethoprim)	Blocking folate biosynthesis by Inhibiting dihydrofolate reductase (DHFR) enzyme which catalyzes the formation of tetrahydrofolic acid from dihydrofolic acid
Mechanisms of resistance	(1) Chromosomal mutation in the *fol*P and *fol*A gene (encoding the DHPS and DHFR enzymes) (2) Efflux pump (e.g. BpeEF-OPrC (in the resistance/nodulation/division superfamily) in *Burkholderia pseudomallei*) (3) Naturally insensitive target enzymes (4) Acquisition of SMX^r and TMP^r variants of the DHPS and DHFR enzymes (e.g. *sul*1, *sul*2))
Mobility of ARGs (sulfamethoxazole)	The occurrence of plasmid-encoded enzymes on integrons (e.g. *sul*1) and small plasmids (e.g. *sul*2)

Mibility of ARGs (trimethoprim)	Cassette-mediated ARGs such as: • Family type I (e.g. enzyme types I, V, VI, VII) in G(-) bacteria • Family type II (e.g. enzyme types IIa, IIb, and IIc)
Antimicrobial	**Amoxicillin** **(Munita and Arias, 2016)** 
Bacterial host range	Broad spectrum including a wide range of G(+) (e.g. *Enterococcus faecalis*) and G(-) (e.g. *E. coli*) bacteria
Classification	β-Lactams (3rd generation of penicillins/aminopenicillins)/semisynthetic/ bactericidal
Clinical usage	Treatment of tonsillitis, bronchitis, pneumonia, skin, ear, nose, throat, skin and urinary and lower respiratory tract infections
Mode of action	Inhibition of the cell wall synthesis by disrupting the function of penicillin-binding proteins (PBPs) enzymes
Mechanisms of resistance (encoded enzymes)	(1) Intrinsic and acquired β-Lactamases located either on chromosomae or plasmids (carrying transposable elements) (e.g. Class C β-Lactamases or penicillinases) (2) Modifications of the PBPs as the target site (e.g. *mec*A gene encoding a PBP) (3) Reducing drug accumulation by decreased uptake or Increased efflux (e.g. in *Klebsiella pneumoniae*)
Mobility of ARGs	The occurrence of • Plasmid mediated β-Lactamases (e.g. extended spectrum beta-lactamase TEM) • Genes encoding efflux pumps on mobile genetic elements (MGEs) (e.g. OXA-2 gene on class 1 integrons)

In Section 3.3, the foam fosmid library was screened for resistance to carbenicillin (CARB). While β-lactam antibiotics are the most prescribed antibiotics worldwide with a large diversity of known ARGs, the prescribing of CARB (in the carboxypenicillin subgroup of penicillins) is limited to cases where resistance to other β-lactams such as amoxicillin (AMX) or ampicillin (AMP) is observed. Like other β-lactams, this antibiotic is used to treat lower urinary tract infections and asymptomatic bacteriuria caused by *Pseudomonas aeruginosa*, *E. coli* and some *Proteus* species. With limited usage of CARB, it is expected to detect a low frequency of resistance to this antibiotic in hospital water and WWTPs. In an effert to identify novel ARGs in the foam fosmid library, CARB was selected to be studied.

Table 2.6 summarizes some properties of target compounds in the present study whose

resistance phenotype in bacterial isolates and/ or fosmid library clones were monitored by growth inhibition testing in liquid or on solid medium. The remainder of the target compounds not included in **Table 2.6** are the antimicrobial agents whose resistance properties in bacterial strains were only tested by disk diffusion assay. Antimicrobial susceptibility test disks were purchased from BBL (BBL, Division of Bioquest, Cockeysville, Mayland, USA).

Table 2.6 List of the target antimicrobials, properties of stock solutions and chemical suppliers

Antimicrobial class	**Target antibiotic**	**Solvent/ sterilization technique**	**Supplier (CAS NO.)**	**Example of encoded enzyme/ ARG Name** (Szczepanowski et al., 2009)
ß-Lactams	**amoxicillin (AMX)**	ddH_2O filter sterilized	Sigma (26787-78-0)	Class D β-lactamase/ bla_{OXA-20}
	carbenicillin (CARB)	ddH_2O filter sterilized	Sigma (4800-94-6)	Class D β-lactamase/bla_{OXA-48}
Fluoroquinolones	**ciprofloxacin (CIP)**	ddH_2O/filter sterilized	MP Biomedicals (86393-32-0)	Pentapeptide family protein/*qnr*A
Sulfonamides	**sulfamethoxazole (SMX)**	Ethanol	Sigma (723-46-6)	DHPS enzyme/*sul*1
Pyrimidines	**trimethoprim (TMP)**	Methanol	Sigma (738-70-5)	DHFR enzyme/*dfrI*
Tetracyclines	**tetracycline (TET)**	15% Ethanol filter sterilized	Sigma (64-75-5)	Tetracycline efflux/*tet*(A)

ddH_2O: distilled deionized water

2.4 Analytical Methods

2.4.1 Culture-based Techniques

Culture-dependent approaches commonly involve enrichment and isolation of target bacteria on selective or non-selective media (McLain et al., 2016). Detection of antimicrobial resistance (AR) in bacteria is performed by evaluating growth properties in response to specific concentrations of antimicrobials on solid or in liquid medium. Although the application of culture-based techniques is limited to a small fraction of microoorganisms in an environment which can be cultivated under laboratory conditions, these techniques can

provide information on the genotypic and phenotypic properties of individual bacterial strains. Major advantages of culture-dependent techniques include the potential to identify the host organisms of antibiotic resistance genes (ARGs), search for multiple drug resistance and study the antimicrobial production properties of target strains (Lin et al., 2015).

In the present research, commonly used antimicrobial susceptibility tests such as agar disk diffusion and measurement of minimum inhibitory concentrations (MICs) (by broth microdilution method and E-tests) were performed. In order to determine the proportion of total cultivable heterotrophic bacteria in different compartments of the MEBPR processes which are resistant to an antimicrobial of interest, two different approaches of bacterial growth inhibition testing in antimicrobial-supplemented liquid or solid medium were applied. Sections 2.4.1.1 to 2.4.1.4 provide a detailed description of the culture-based methodologies used in this study.

2.4.1.1 Antimicrobial Susceptibility Tests

Susceptibilities of bacterial isolates to antimicrobials were determined by the standard disk diffusion method, according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2011). In detail, liquid cultures were first prepared from purified colonies by restreaking the isolates twice and verifying their purity by microscopy. When the inoculum density of broth cultures reached 0.5 MacFarland turbidity standard (in mid-logarithmic phase of growth), a cell suspension was spread on MH-II agar plates with sterile cotton swabs three times (rotating the plate approximately 60° each time to obtain uniform growth). Antibiotic disks were then placed on inoculated agar plates and incubated for 16-18 hours at 37°C before the diameter of the zone of inhibition was measured. Data representing the zone of inhibition are commonly interpreted using guidelines which classify clinical isolates to “susceptible”, “resistant” or “intermediate” (CLSI 2011, EUCAST 2011). However, as target isolates in the present project contained a large diversity of bacteria whose identity was not known at the species level, data analysis was not possible based on published standards. Hence, a new criterion was defined to analyze the data. In this study, isolates were defined as resistant when the zone of inhibition was less than one centimeter so that

the possibility of reporting false positive results for resistance could be minimized. When a bacterial isolate exhibited resistance to antimicrobials from at least three different classes, it was considered multidrug-resistant (MDR) (Mokracka et al., 2012). It should be remembered that if bacterial isolates (strain R2A-P9 (Section 3.2.5.2)) could not grow on MH-II agar plates, the initial isolating medium (R2A) was used in disk diffusion assay testing.

The minimum inhibitory concentrations (MICs) of antibiotics against some of the bacterial isolates of this project were determined using the standard two-fold serial broth microdilution method (CLSI, 2011) covering a concentration range of 1.95 to 2000 ng/µL in MH-II medium. As a dilution-based growth inhibition assay, MIC is defined as the lowest concentration of an antimicrobial compound which inhibits the visible growth of bacterial isolates (Andrews, 2001).

Another approach to determine the MIC of antibiotics against some bacterial isolates was the E-test method. Briefly, a suspension of the test organism (turbidity equivalent to 0.5 MacFarland turbidity standard) was spread on MH-II agar plates (streaking in three directions over the entire surface) using a sterile cotton swap. Antimicrobial plastic strips (bioMérieux, Marcy-l'Etoile, France) were then applied onto the agar plate and incubated at 37°C for 16-18 hours. As test strips consist of a linear gradient of antimicrobial concentrations, a symmetrical inhibition ellipse centered along the strip provides an accurate measurement of MIC.

2.4.1.2 Enumeration of Antimicrobial-resistant Heterotrophic Bacteria

2.4.1.2.1 Bacterial Growth on Solid Media

Bacteriological counts of total and resistant cultivable heterotrophs in influent, mixed liquor and foam samples were performed by spread plate method as outlined in standard method 9215C (Eaton et al., 2005). Briefly, samples were homogenized, ten-fold serially diluted in phosphate buffered dilution water (0.0425 g/L KH_2PO_4 and 0.0405 g/L $MgCl_2.6H_2O$) and plated in triplicate by sterilized L shaped spreaders on agar plates. After incubation, the number of colonies on each plate presenting between 50 to 200 cells was manually counted and colony forming units (CFUs) per sample volume (influent and mixed liquor) or per

sample weight (foam) were recorded. For effluent samples, the membrane filtration technique was applied using standard microbiological procedures (Eaton et al., 2005). Briefly, after vacuum filtering the sample through 0.45 µm pore size cellulosic membrane filters (Fisher Scientific, Hampton, New Hampshire, USA), retained particles were placed on agar plates in triplicate and incubated. Calculation of CFU/mL was performed by counting the number of effluent colonies on plates presenting between 20 to 100 typical colonies.

Isolation and enumeration of heterotrophic bacteria in the present project were carried out on two non-selective non-differential media, MH and R2A. MH is the standard nutrient-high medium commonly used for antimicrobial susceptibility testing of fast-growing non-fastidious microorganisms (Matuschek et al., 2014). While low levels of thymine and thymidine in MH medium minimize the occurrence of false identification of resistance in antimicrobial susceptibility testing against sulfonamides and TMP, the adjusted amounts of magnesium and calcium reduce the effects of variation in divalent cations in the aminoglycoside and TET tests (Ferguson and Weissfeld, 1984). MH medium contains starch (1.5 g/L), casamino acids (17.5 g/L) as well as beef infusion (2 g/L) which provide the nutrient supply and energy to yield good bacterial growth. MH broth was purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

R2A (a nutrient-low medium) was introduced by Reasoner and Geldreich (1985) for bacteriological plate counts of treated potable water to stimulate the growth of stressed and chlorine-tolerant bacteria over long incubation times. R2A is now on the list of standard media used to culture slow- and fast-growing heterotrophic bacteria using spread plate, pour plate and membrane filter methods (Eaton et al., 2005). Many studies in recent years have used this medium to isolate total heterotrophic bacteria for studies of AR in samples of WWTPs (Zhang et al., 2015b; Aali et al., 2014; Oh et al., 2009; Gao et al., 2012; Garcia-Armisen, 2011; Kim et al., 2007b). R2A medium (Difco) contains yeast extract (0.5 g/L) as a source of vitamins and trace elements, proteose peptone No.3 (0.5 g/L) and casamino acids (0.5 g/L) to provide minerals, carbon, nitrogen, vitamins and amino acids, dextrose (0.5 g/L) as a carbon source, starch (0.5 g/L) to protect against toxic materials, sodium pyruvate (0.3 g/L) to increase the recovery of stressed cells, dipotassium phosphate (0.3 g/L) to provide phosphate and balance pH and magnesium sulfate (0.05 g/L) as a source of divalent cations and sulfate (Zimbro et al., 2009).

To ensure the optimal growth of slow-growing heterotrophs, R2A plates were incubated at 37°C for 24 hours and then placed at room temperature for up to one week prior to counting CFUs unless otherwise is stated. Colony counting on MH plates was done after 16-18 hours of incubation at 37°C. To avoid fungal growth, both agar media were supplemented with cycloheximide at a concentration of 75 µg/mL. As the focus of the present project is on evaluating the potential of release of ARB from MEBPR systems concerning human health, 37°C was chosen as the incubation temperature for the growth of heterotrophic bacteria in media (Allen et al., 2004).

2.4.1.2.2 Bacterial Growth in Liquid Media

Section 3.2 compares the ARB and MDR patterns of heterotrophic bacteria in influent and two MEBPR processes operating at either 25 or 60 day SRTs. As monitoring the MDR patterns required each bacterium to be screened individually against a set of antimicrobials of interest, simple plating of bacterial cells on antimicrobial supplemented agar plates could not be used. Hence, to provide an opportunity for simultaneous screening of bacteria for resistance to antimicrobials, bacterial colonies were first isolated and the glycerol stocks were stored for further testing. In order to isolate bacteria, colonies were stabbed with sterilized toothpicks and suspended in 80 µL of initial liquid medium (MH or R2A) containing 10% glycerol in 384-well microtiter plates, incubated and stored at -80°C. To minimize the potential of cross contamination, overlapping but still distinguishable colonies were not isolated. Single colonies were isolated from samples of influent, anoxic mixed liquor (25 and 60 day SRTs), treated effluents (25 and 60 day SRTs) as well as foam (25 and 60 day SRTs) collected on December 2014, January 2015 and February 2015. As previous work by Monti (2006) and Lawson (2014) showed that the UBC pilot plant redox reactors behaved as completely mixed tanks, colonies isolated from anoxic tanks were considered to be representative of the total community of heterotrophic bacteria in the redox reactors.

Before antimicrobial screening, plates corresponding to the first replicate of each sample were thawed and replicated into brand new 384-well microtiter plates using a QPix2 Robotic colony picker. After incubation (37°C for 16-18 hours in MH and 36-38 hours in R2A medium), optical density (OD) of the fresh cultures was measured at 600 nm by a Varioskan

Flash spectral scanning plate reader.

Screening was conducted by replicating fresh cultures into 384-well microtiter plates supplemented with desired concentrations of antimicrobials in the liquid media. Methodology applied for these experiments was a novel application of the QPix2 Robotic colony picker technology. Using this approach, each isolated colony was screened in duplicate once by the QPix2 Robotic colony picker and once by a 384 pin multi-blot replicator for resistance to a set of five antimicrobials, SMX (50 µg/mL), TMP (5 µg/mL), AMX (32 µg/mL), TET (10 µg/mL) and CIP (5 µg/mL). Screening plates were bleached, ethanoled (80%) and exposed to ultraviolet (UV) irradiation to avoid contamination. The protocol for bleach/ethanol/UV sterilization was verified to test for the cleanliness of the 384-well microtiter plates (data not shown). One unused plate was also designated as a positive control to monitor the viability of cells in antimicrobial-free media in parallel with antimicrobial screening assay. **Table 2.5** summarizes some general properties of the tested antimicrobials in the present section and their corresponding resistance mechanisms.

After incubation, the OD_{600} of bacterial cultures in screening plates was measured. Organisms whose growth in the presence of the desired concentration of an antibiotic was confirmed in duplicate plates, as well as the positive control plate were considered to be resistant. To optimize the inoculum suspension density (within the range of 2 to 8×10^5 CFU/mL following the CLSI (2011) guidelines using broth microdilution techniques), the QPix2 Robotic colony picker was set for a single dip and two seconds of pin contact time in the destination plates. This setting was confirmed by measuring the inoculum density of 50 cell cultures (25 MH- and 25 R2A-isolated strains randomly selected from two of 384-well microtiter positive control plates) using the serial dilution plating method. In another experiment, the inoculum densities of 20 cell cultures (10 MH- and 10 R2A-isolated strains selected from 20 different 384-well microtiter plates) whose difference in OD_{600} measurements before and after inoculation (before incubation) was highest were measured. Data showed that the difference (> 0.01) between OD_{600} values before and after inoculation could be used as a useful parameter to minimize the possibility of including bacteria with inoculum densities above the recommended range in screening results. Using this approach, about 1.2% of total tested bacterial isolates with suspected inoculum densities above 8×10^5 CFU/mL were removed from the data analysis.

In total, from each environment (influent, anoxic mixed liquor (SRT = 25 and 60 days), foam (SRT = 25 and 60 days) and treated effluent (SRT = 25 and 60 days)), an average of 200 MH- and 150 R2A-isolated bacteria (from each of the three sampling events) were screened for resistance to five antimicrobials.

2.4.1.2.3 Data analysis

In order to determine the ARB ratios of cultivable heterotrophic bacteria on solid media, the total number of bacterial colonies growing on medium supplemented with antimicrobials was divided by colony counts of bacteria growing on medium without antimicrobials. In liquid assays, ARB ratios were determined by dividing the total number of organisms showing positive growth in antimicrobial supplemented liquid media by the total number of viable organisms in antimicrobial-free media (positive control plate). When reported as percentage, ARB ratio was multiplied by 100. Multiplying the ARB ratio by colony counts of the same sample (CFU/mL), the concentration of resistant bacteria (CFU/mL) in each sample was determined.

Statistical pair-wise comparisons were made assuming a simple t-based hypothesis test between two means on the basis of media, populations and antibiotics at a significance level of 0.05. Using this method, the statistical significance between two mean ratios of ARB as well as MDR bacteria (i.e. bacterial resistance to at least three antimicrobials from three different classes) were compared when the sample size was a minimum of three. Hence, the usage of the word “significance/significant” is only limited to cases where statistical analysis was performed on data (e.g. **Appendix B** (**Table B.2** to **B.4**)).

Percent removal of total heterotrophic bacteria as well as ARB by membrane filtration was calculated from the following formula:

$$Percent\ removal = \frac{C_{influent} - C_{effluent}}{C_{influent}} \times 100 \quad (1)$$

$C_{influent}$: concentration of total heterotrophic bacteria, ARB and/ or ARG in influent (CFU/mL)

$C_{effluent}$: concentration of total heterotrophic bacteria, ARB and/ or ARG in treated effluent (CFU/mL)

Log reduction of total heterotrophic bacteria, ARB, as well as ARG, through membrane filtration was calculated from the following formula:

$$Log\ reduction = Log_{10}\ (\frac{C_{influent}}{C_{effluent}}) \quad (2)$$

The inflow rate of ARB in influent as well as release rates (RR) of ARB in treated effluent, waste aerobic mixed liquor and foam were calculated from the following formulae:

$$IR = C_{influent} \times Q_{influent} \quad (3)$$

IR: inflow rate of ARB (CFU/day) or input ARG concentration (gene copies/day) to MEBPR processes

$C_{influent}$: concentration of ARB (CFU/L) or ARG (gene copies/L) in influent

$Q_{influent}$: influent daily flow rate (L/day)

$$RR_{waste\ aerobic\ mixed\ liquor} = C_{aerobic\ mixed\ liquor} \times Q_{waste\ aerobic\ mixed\ liquor} \quad (4)$$

$RR_{waste\ aerobic\ mixed\ liquor}$: release rate of ARB in waste aerobic mixed liquor (CFU/day)

$C_{aerobic\ mixed\ liquor}$: concentration of ARB in aerobic mixed liquor (CFU/L)

$Q_{waste\ aerobic\ mixed\ liquor}$: aerobic mixed liquor wastage rate (L/day)

$$RR_{foam} = C_{foam} \times Q_{foam} \quad (5)$$

RR_{foam}: release rate of ARB in foam (CFU/day)

C_{foam}: concentration of ARB in foam (CFU/kg)

Q_{foam}: foam wastage rate (kg/day)

$$R_{effluent} = C_{effluent} \times Q_{effluent} \quad (6)$$

$RR_{effluent}$: release rate of ARB through the discharge of MEBPR effluent (CFU/day) or release rate of ARG through the discharge of MEBPR effluent (gene copies/day)

$C_{effluent}$: concentration of ARB (CFU/L) or ARG (gene copies/L) in membrane effluent

$Q_{effluent}$: outflow rate (L/day)

The total release rate of ARB was calculated from the following formula:

$$RR_{total} = RR_{waste\ aerobic\ mixed\ liquor} + RR_{foam} + RR_{effluent} \quad (7)$$

The fraction (F_{ARB}) of contribution of waste aerobic mixed liquor, foam and MEBPR effluent, in the release of ARB was calculated from the following formulae (Munir et al., 2011):

$$F_{ARB\ (aerobic\ mixed\ liquor)} = \frac{RR_{aerobic\ mixed\ liquor}}{IR} \quad (8)$$

$F_{ARB\ (aerobic\ mixed\ liquor)}$: fraction of contribution of waste aerobic mixed liquor in the release of ARB

$$F_{ARB\ (foam)} = \frac{RR_{foam}}{IR} \quad (9)$$

$F_{ARB\ (foam)}$: fraction of contribution of foam in the release of ARB

$$F_{ARB\ (effluent)} = \frac{RR_{effluent}}{IR} \quad (10)$$

$F_{ARB\ (effluent)}$: fraction of contribution of treated effluent in the release of ARB or ARG

In this study, the standard deviation (σ) and standard error of the mean (σ_M) were calculated from the following formulae:

$$\sigma = \sqrt{\frac{\sum(x-\bar{x})^2}{n-1}} \quad (11)$$

x: each value

$\bar{x}$: the average of all values

n: sample size

$$\sigma_M = \frac{\sigma}{\sqrt{n}} \quad (12)$$

The composite standard deviation (σ R) was calculated from the following formulae:

If $R = \bar{y}/\bar{z}$

$$\sigma R = R\sqrt{(\frac{\sigma\bar{y}}{\bar{y}})^2 + (\frac{\sigma\bar{z}}{\bar{z}})^2} \quad (13)$$

$\bar{y}$ and $\bar{z}$: the average values of two sets of independent samples

$\sigma\bar{y}, \sigma\bar{z}$: standard deviation of indepenedent samples (y and z)

If $R = \bar{y} + \bar{z}$

$$\sigma R = \sqrt{(\sigma\bar{y})^2 + (\sigma\bar{z})^2} \quad (14)$$

2.4.1.3 Lactose Fermentation Test

To compare the proportion of lactose fermenting (Lac+) bacteria between MH- and R2A-isolated effluent heterotrophs, the growth properties of 96 MH and 96 R2A bacterial isolates on MacConkey solid medium were studied. This assay was performed on MH and R2A heterotrophic bacteria isolated from membrane effluent samples collected on December 2014 and January 2015 from a 25 day SRT MEBPR train which were tested for resistance to five antimicrobials by growth in liquid medium (methodology provided in Section 2.4.1.2). To ensure colonies were representative of the total community of bacteria which had been screened for resistance to antimicrobials (Section 3.2), bacterial isolates with similar patterns

of resistance to the total previously tested effluent bacteria were randomly selected. Lactose fermentation tests were performed by transferring the cells from fresh liquid cultures (prepared in 96 well plates in MH or R2A media) by a 96 pin multi-blot replicator to MacConkey agar uni-well rectangular plates in duplicate. After proper incubation (**Table 2.7**), enteric bacteria with the ability to utilize lactose were detected by the appearance of pink colonies due to lactose fermentation and acid production.

MacConkey agar is a selective and differential medium used to isolate G(-) bacteria and separate them based on their ability to ferment lactose. Briefly, peptone (17g/L) and proteose peptone (3 g/L) provide amino acids; vitamins and nitrogenous compounds are required for bacterial growth; lactose monohydrate (10 g/L) is added as a fermentable source of carbohydrate for energy; crystal violet (0.001 g/L) and bile salts (1.5 g/L) act selectively to inhibit the growth of most G(+) bacteria; sodium chloride (5 g/L) is incorporated to maintain the osmotic balance and neutral red (0.03 g/L) is a pH indicator. In short, differentiation of enteric bacilli in MacConkey agar is achieved by utilizing lactose in Lac+ bacteria and acid production, which turns the neutral red pH indicator from colorless to red and precipitates the bile salts in the immediate neighborhood of the colony (MacConkey, 1908).

As eosin methylene blue (EMB) has similar differentiation properties to MacConkey medium (**Table 2.7**), growth properties of the tested bacteria on EMB medium was used as a control to confirm the population of lactose fermenting (Lac+) bacteria, Gram negative (G(-)) non-lactose fermenting (Lac-) bacteria and potential Gram positive (G(+)) bacteria. Parallel experiments were also performed on the initial medium (MH or R2A) to serve as a positive control to test the viability of cells.

Table 2.7 Properties of lactose ferming and G(-) non-lactose fermenting bacetrial growth on MacConkey and EMB media

Medium	Selectivity/ Differentiability Properties	Colonial Characteristics	Incubation Conditions
MacConkey	selective for G(-) bacteria differential for Lac+ bacteria	➢ colorless if G(-) Lac- bacteria ➢ red to pink if Lac+ bacteria	18-24 hours @ 37°C
EMB	selective for G(-) bacteria differential for fecal coliforms and *E. coli* strains	➢ uncolored or light-pink if Lac- and weak Lac+ bacteria ➢ dark blue/purple if strong Lac+ bacteria ➢ dark blue/purple with metallic green sheen if *E. coli* strains	18-24 hours @ 37°C

2.4.2 Molecular Techniques

The application of nucleic acid-based approaches in AR studies overcomes the limitations of culture-dependent techniques in many aspects. As an example, molecular techniques can be used for detection of ARGs in isolated bacteria or the mixed environmental DNA including slow-growing and unculturable microorganisms (Zhang and Zhang, 2011; Ramsden et al., 2010). Culture-independent techniques are also commonly used to quantify AR determinants in environmental samples (Xia et al., 2012; Lachmayr et al., 2009). Through recent advances in metagenomics, function-based analysis of microbial communities has enabled identification of the diversity of unidentified ARGs with unknown sequences in environmental microbiomes (Parsley et al., 2010).

In the present project, culture-independent techniques were applied for four major purposes of (1) detection and quantification of ARGs, (2) identification of influent and treated effluent bacterial isolates by 16S rRNA gene sequencing, (3) profiling the taxonomic composition of bacterial communities in influent, mixed liquor, foam and effluent, and (4) functional screening of the foam fosmid library. Sections 2.4.2.1 to 2.4.2.7 provide a detailed description of the molecular techniques applied in this study.

2.4.2.1 DNA Extraction and Quantification

This section describes the methods for DNA extraction from influent, mixed liquor, foam and membrane-treated effluent samples used for PCR, qPCR and 16S rRNA gene sequencing of bacterial isolates as well as total genomic DNA. The procedures of plasmid and genomic DNA extraction and following purification steps in study of metagenomic libraries are provided in a separate section (2.4.2.5).

In the present research, total genomic DNA extraction from samples of treated effluent was carried out following the protocol by Wright and coworkers (2009). Total genomic DNA in influent, mixed liquor and foam was extracted using the FastDNA® Spin Kit for soil (QBiogene Mississauga, Ontario, Canada). Isolated DNA was examined by gel electrophoresis (1.0% (wt/vol) agarose gel in TAE buffer) and ethidium bromide (EtBr) staining.

In the present study, a total of 72 (16 influent and 56 membrane effluent) bacterial isolates were identified by 16S rRNA gene sequencing. As a ubiquitous component of ribosomes, the 16S rRNA gene is used as the most suitable gene target to study bacterial phylogeny and diversity. The 16S rRNA gene consists of nine hypervariable regions (V1-V9) with each region flanked by a conserved segment (Yang et al., 2016). While these highly conserved regions enable amplification of the 16S rRNA gene by broad-range (universal) primers in widely divergent microorganisms, sequencing the hypervariable regions allows superior phylogenetic resolution. In the present research, PCR reactions were perfomed using crude bacterial cell lysates as template DNA. In detail, 200 µL of the overnight bacterial culture was first centrifuged at 7000 g for two minutes. After the supernatant was discarded, 100 µL of nuclease-free sterile water was added to the tube and mixed well. The solution was boiled for 20 minutes and then centrifuged for two minutes at 14000 g. The supernatant was carefully collected in a sterilized microcentrifuge tube and stored at -20°C until processing.

In cases the plasmid or genomic DNA was required to be concentrated, the ethanol precipitation procedure was followed. Briefly, DNA was first mixed with 3M NaOAc (0.1 × DNA volume) and 100% ethanol (2 × total volume of DNA and 3M NaOAc) and centrifuged for two minutes at 4000 g. After the supernatant was discarded, the pellet was resuspended

in 200 µL of 70% ethanol to wash the remaining salt in the solution. The solution was centrifuged for two minutes at 4000 g, the pellet was vacuum dried and resuspended in 25 µL of nuclease-free sterile water.

In the present project, the concentration of DNA was measured using two different methods. Estimation of purity and concentration of nucleic acids in 16S rRNA gene amplified products were measured by microspectrophotometery (NanoDrop ND-1000, NanoDrop Technologies, Wilmington, Delaware, USA). Absorbance was measured at wavelengths of 260 (A260) and 280 (A280) nm. DNA with absorbance quotient (OD_{260}/OD_{280}) between 1.8 and 2.0 was considered purified.

Accurate quantification of double stranded DNA in influent and MEBPR samples was performed by fluorescence staining using the Quant-iT™ PicoGreen® dsDNA Kit (Invitrogen, USA). In the first step, three dilutions of the sample (1:50, 1:100 and 1:1000) were prepared in duplicate. In order to make a calibration curve, serial dilutions of the standard (Lambda DNA) were prepared in triplicates at eight gradient concentrations ranging from 15.6 to 2000 ng/µL. Then, 25 µL of the solution in each tube (diluted samples and/or standards) was mixed with 25 µL of diluted picogreen fluorescent nucleic acid stain (1:200 dilution in 1× TE buffer) in duplicate in a 96-well microtiter plate. As the picogreen reagent is susceptible to photodegradation, the plate was kept in the dark and the fluorescence of standards and samples was measured within 10 minutes of the addition of picogreen reagent using a Varioskan Flash Spectral Scanning Plate Reader (Thermo Scientific, Waltham, Massachusetts, USA) at 535 nm emission and 485 nm excitation.

2.4.2.2 PCR and Amplicon Detection

In the present project, qualitative PCR assays were performed for two purposes of (1) studying the phylogeny of influent and effluent isolated bacteria (by 16S rRNA gene sequencing analysis) and (2) detection of ARGs in bacterial isolates and total genomic DNA in different compartmanets of the MEBPR process. A PCR reaction master mix was composed of 4 µL of reaction buffer-Mg^{2+} (10×), 1.2 µL of 50 mM $MgCl_2$, 0.9 µL of dNTP mixture (10 mM), 0.2 µL of Taq polymerase (5 U/µL), 1.5 µL of each primer (10 µM) and 25.7 µL of nuclease-free sterile water. The reaction mixture was mixed with 1 µL of template

DNA (~ 1 ng) and PCR amplification was performed with a 2720 Thermal Cycler (Applied Biosystems, California, USA). **Table 2.8** provides the details of PCR conditions. **Table 2.9** summarizes the sequences of primers used in PCR and qPCR assays

Products of PCR amplification were analyzed by gel electrophoresis (in 1% (wt/vol) agarose in 0.5 × TAE buffer) stained with EtBr and visualized using UV gel dock so that the size of the amplified product could be compared with the control. In case the positive control was not available but the desired size of the amplified product was confirmed by gel visualization, PCR products were cleaned up following the isopropanol precipitation procedure before sequencing. The procedure is similar to ethanol precipitation (Section 2.4.2.1) with the exception that PCR products were mixed with NaOAc (0.1 × PCR product volume) and Isopropanol (0.7 × PCR product volume) before initial centrifugation.

Table 2.8 PCR reaction conditions

Target Gene	Initial Denaturation		No. of cycles	Denaturation		Annealing		Elongation		Final Extension	
	°C	Minutes		°C	Seconds	°C	Seconds	°C	Seconds	°C	Minutes
*aad*A1	95	5	35	95	20	54.0	30	72	30	72	5
bla_{OXA-2}	95	7	35	95	20	56.2	20	72	120	72	5
bla_{TEM-1}	95	7	40	94	60	58.0	30	72	30	72	7
Class 1 Integron variable region	94	5	25	94	30	60.0	30	72	30	72	7
*int*1	94	5	25	94	30	60.0	30	72	30	72	7
*sul*1	95	5	40	95	30	55.9	30	72	30	72	7
*sul*2	95	5	40	95	30	60.8	30	72	30	72	7
tet(A)	95	5	35	95	20	55.2	30	72	30	72	7
tet(C)	95	5	35	95	20	55.2	30	72	30	72	7
tet(G)	95	5	35	95	20	62.0	20	72	30	72	5
tet(W)	95	5	35	95	20	60.4	20	72	30	72	5
16S rRNA	94	3	35	94	40	55.0	90	72	120	72	10

Table 2.9 Oligonucleotides used in this study for PCR and qPCR assays

Target	Sequences (5'-3')		Amplicon Size	Application	Reference
*aad*A1	F-K185	CCGAAGTATCGACTCAAC	747	PCR	Davies Laboratory
	R-K186	CGACTACCTTGGTGATCT			
bla_{OXA-2}	F-K176	AAGAAGGCACGCTAGAAC	640	PCR	Davies Laboratory
	R-K177	AGTGCGAAGAATACGGAG			
bla_{TEM-1}	F	CATTTTCGTGTCGCCCTTAT	167	PCR qPCR	Yang et al., 2012
	R	GGGCGAAAACTCTCAAGGAT			
Class 1 Integron variable region	hep58-K459	TCATGGCTTGTTATGACTGT	variable	PCR	Malek et al., 2015
	help59-K460	GTAGGGCTTATTATGCACGC			
*int*1	F-K159	GTTCGGTCAAGGTTCTGG	890	PCR	Xu et al., 2007b
	R-K160	CGTAGAGACGTCGGAATG			
*sul*1	F	CGCACCGGAAACATCGCTGCAC	163	qPCR	Pei et al. (2006)
	R	TGAAGTTCCGCCGCAAGGCTCG			
*sul*2	F-K286	TTGGGGCTTCCGCTATTGGTCT	187	PCR	Nandi et al., 2004
	R-K287	GGGTTTCCGAGAAGGTGATTGC			
*sul*2	F	TCCGGTGGAGGCCGGTATCTGG	191	PCR	Pei et al. (2006)
	R	CGGGAATGCCATCTGCCTTGAG			
*tet(*A)	F	GCTACATCCTGCTTGCCTTC	210	PCR	Ng et al., 1999
	R	CATAGATCGCCGTGAAGAGG			
tet(C)	F	CTTGAGAGCCTTCAACCCAG	418	PCR	Davies Laboratory
	R	ATGGTCGTCATCTACCTGCC			
*tet(*G)	F	GGTGCTTCTGGCTTCTCTTG	140	qPCR	Szczepanowski et al. (2009)
	R	CAATGGTTGAGGCAGCTACA			
*tet(*W)	F	GTCGAAAAAGGGACAACGAG	172	qPCR	Szczepanowski et al. (2009)
	R	CTAAAACAGCCAAAGAGCGG			
16S rRNA	7F	GAGAGTTTGATCCTGGCTCAG	1504	PCR	Srivastava et al., 2008 Zhong et al., 2010
	1511R	CGGCTACCTTGTTACGACTTC			
16S rRNA	27F	AGAGTTTGATCCTGGCTCAG	492	qPCR	Zaikova et al., 2010
	519R	GNTTTACCGCGGCKGCTG[a]			
K560		TGCCACAACTATCGTGCCTT	-	sequencing	this study
K561		CCGTGCCTTTTCGCAGTTAG	-	sequencing	this study
K562		AGAACAAGCAGGCATCACGA	-	sequencing	This study

[a] International Union of Pure and Applied Chemistry (IUPAC) degenerate base symbols; N=A, G, C, or T; K=G or T.

Purified products were sent to either Genewiz (South Plainfield, New Jersey, USA) or Macrogen (Geumcheon-gu, Seoul, Korea) or the Nucleic Acid Protein Service Unit (NAPS UBC, Vancouver, Canada) for DNA sequencing. Sequencing data were analyzed using BioNumerics Software (v.5.10) and phylogenetic trees were assembled by the neighbour joining method in macVector (v.13.0). Basic local alignment tool (BLAST) was used for rapid comparison of nucleotide sequences.

2.4.2.3 Quantitative Real-time PCR Assays

The relative abundances of the *sul*1 gene in influent and MEBPR processes was determined by real-time quantitative PCR (qPCR) assay testing. To correct for variations in the efficiency of DNA extraction, the number of *sul*1 gene copies in each sample was normalized to the corresponding 16S rRNA gene copies as a surrogate measure of total bacterial abundances (Devarajan et al., 2016). The procedure for qPCR assays was developed using the SsoFast EvaGreen Supermix (BioRad, Hercules, Canada). The reaction mixture was composed of 10 µL of Ssofast EvaGreen supermix, 1 µL of each primer (10 µM), 6 µL of sterile RNase/DNase-free water and 2 µL of template DNA (1:10 dilution). Fluorescence detection and thermal cycling were conducted on a CFX96 real-time PCR detection system and CFX Manager software (v.3.1; BioRad), using the following protocol: 98°C for two minutes, followed by 39 cycles of 98°C for two seconds and five seconds at the annealing temperature (55 °C for 16S rRNA gene, 65 °C for *sul*1 gene) followed by a melt curve stage with temperature ramping from the annealing temperature to 95 °C. In order to establish the optimal annealing temperature to amplify the *sul*1 gene, gradient PCR was first performed.

In order to generate the calibration curve for quantification of the *sul*1 gene, purified PCR products (confirmed by sequencing in this study) were used as the standard. The standard for 16S rRNA gene quantification, cloned into *Escherichia coli* (clone ID: 215EB), was kindly provided by Dr. Steven Hallam (Professor at UBC, Department of Microbiology and Immunology, personal communication). The concentrations of DNA in the standards were measured by picogreen assay testing (methodology provided in Section 2.4.2.1). Ten-fold serial dilutions of the plasmid DNA or PCR products were used to make the calibration curves. The following formula correlates the concentration of DNA to gene copy numbers:

$$\text{DNA}\left(\frac{\text{copies}}{\mu\text{L}}\right) = \frac{6.022\times10^{23}\left(\frac{\text{copies}}{\text{mole}}\right)\times\text{DNA concentration}\left(\frac{\text{ng}}{\mu\text{L}}\right)}{\text{Fragment size (bp)}\times660\ (\text{g/(mole.bp)}\times10^{9}\left(\frac{\text{ng}}{\text{g}}\right)} \quad (15)$$

Samples were analyzed in triplicate with a negative control and a standard curve in each run. Within each assay, the gene copy numbers of replicate pairs were averaged. In order to minimize the error due to repeated freeze thawing, DNA was stored in small aliquots (at -20°C), so that each tube of DNA was only thawed once. To reduce human and instrumental error, one set of calibrated pipettes was used for all qPCR reactions. The amplification efficiency (E) of qPCR reactions ranged from 89.8% to 96.7% and correlation coefficient (R^2) values were more than 0.998 in all calibration curves. The specificity of qPCR products was checked by agarose gel electrophoresis and melt curves.

2.4.2.4 Profiling the Taxonomic Composition of Bacterial Communities through High-throughput Sequencing of the 16S rRNA Gene

The composition and relative abundances of bacterial communities involved in a MEBPR system operating at 25 day SRT were determined by Illumina high-throughput sequencing. As a preparatory step, the concentration of total genomic DNA extracted from samples of influent, foam, membrane-treated effluent, anaerobic, anoxic and aerobic mixed liquors collected on December 2014, January and February 2015 were measured by picogreen assay. The procedures for extraction and quantification of DNA were described in Section 2.4.2.1. Then, equal concentrations of DNA from each of the individual samples of influent, mixed liquor (anaerobic, anoxic, aerobic reactors), foam and treated effluent at three sampling days were pooled together and submitted to Microbiome Insights Inc. (Vancouver, Canada) for PCR, sequencing and partial sequencing analysis. Briefly, PCR reactions were performed using ThermoFisher Phusion Hot Start II DNA polymerase. A reaction master mix was composed of 10 µL of reaction buffer (5 ×), 1 µL of 50 mM $MgCl_2$, 1 µL of dNTP mixture, 0.5 µL of Taq polymerase (2U/µL), 1 µL of each primer (10 µM) and 33.5 µL of nuclease-free sterile water. The reaction mixture was mixed with 2 µL of template DNA. The PCR reaction

was performed using the following protocol: 98°C for two minutes, followed by 30 cycles of 98°C for 20 seconds and 55 °C for 15 seconds and 72 °C for 30 seconds, followed by 72°C for 10 minutes. DNA extracts were sequenced using Read 1 primer (5′-TATGGTAATTGTGTGCCAGCMGCCGCGGTAA-3′), Read 2 primer (5′-AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3′) and Index primer (5′-ATTAGAWACCCBDGTAGTCCGGCTGACTGAC-3′) annealing to the V4 region region of 16S rRNA genes. Sequencing was performed on a MiSeq using the MiSeq 500 Cycle V2 Reagent Kit (Illumina Inc, USA).

MiSeq generated Fastq files were filtered and clustered into operational taxonomic units (OTUs) using Mothur software (v.1.38) following the MiSeq SOP. Briefly, sequence pairs were merged into contigs and quality filtered by removing long homopolymers and ambiguous bases before pre-clustering by merging sequences that differ by less than two bases. Chimera detection was done with UCHIME. Chimeric sequences were removed prior to taxonomic assignment via Bayesian classification. Filtered sequences were clustered into 97% similarity OTUs using the average neighborhood approach into genus level taxa. This part was performed by the Microbiome Insights Inc. (Vancouver, Canada).

As a summary, Illumina 250-bp paired-end sequencing of the amplicon targeting the V4 region of the 16S rRNA gene generated 20,585, 21,805, 18,108 and 19,469 sequencing reads from samples of influent, mixed liquor, foam and membrane effluent, respectively.

After the taxonomic assignment and to reduce the over-prediction of rare OTUs, OTUs represented by one read (singletons) were removed and 1,152 OTUs corresponding to a total of 7,996, 9,305, 6,680 and 7,859 joined reads were retained for downstream microbial community analysis of influent, mixed liquor, foam and membrane effluent, respectively.

After the table of OTUs was prepared, bubble plots of order level abundance within communities were drawn for three classes of OTUs using R packages. Abundant, intermediate and rare OTUs were defined as having a relative abundance or a frequency of > 1%, a frequency between 1% and 0.1% and a frequency < 0.1%, respectively (Galand et al., 2009).

Hierarchal dendrograms were generated using Ward's minimum variance method and Manhattan distance measures. Hierarchal cluster analysis of bacterial communities was

performed using the pvclust package in the R software. Statistically significant similarity between the resulting clusters was calculated as bootstrap core distributions with 1000 iterations and assigned to clusters with bootstrap probability (BP) > 70% and approximately unbiased (AU) > 95%.

Shannon index was calculated using the 'vegan' and 'Phyloseq' R packages, using plot_richness() function. The plot_richness() function generates both a plot, and the appropriate diversity score for each sample in the R data frame. The graphics were generated using ggplot2 package in the R software.

2.4.2.5 Construction of Fosmid Libraries

Metagenomic libraries were constructed from genomic DNA extracted from samples of influent, foam, anaerobic, anoxic and aerobic mixed liquors collected on August 2014 from a MEBPR system operating at 25 day SRT. Isolation of genomic DNA was followed by the protocol proposed for "extracting high molecular weight genomic DNA suitable for constructing large insert environmental metagenomic libraries" by Lee and Hallam (2009). This protocol involves a freeze-grinding step that can improve the cell lysis prior to DNA extraction (Lam et al., 2015). As a representative of the redox zones, equal concentrations of DNA from the anaerobic, anoxic and aerobic mixed liquors were pooled after nucleic acid extraction.

As construction of the metagenomic libraries requires high quality DNA, extracted DNA was treated with RiboShredder RNase Blend (Epicentre, Wisconsin, USA) post extraction to degrade unwanted RNA. In order to remove the remaining proteins, cesium chloride (CsCl) gradient ultracentrifugation was performed following the protocol of Wright and coworkers (2009). Genomic DNA bands illuminated by blue light after CsCl-EtBr gradient centrifugation are shown in **Appendix D**. After DNA purification, the CopyControlTM fosmid library production kit protocol (Epicentre, Wisconsin, USA) was applied to construct fosmid libraries.

In detail, purified insert DNA was first blunt-ended and phosphorylated so that incompatible or damaged 5′ and/or 3′ ends were repaired. Blunt-ended DNA was fractionated by pulse field gel electrophoresis (PFGE) on a 1% low melting point (LMP) agarose gel. The gel slice

between 25 and 45 kb was then extracted from the gel using the GELase enzyme according to the manufacturer's instructions (Epicentre, Wisconsin, USA). After size selection and recovery of the size-fractioned DNA, blunt-ended DNA fragments were ligated into the pCC2FOS fosmid vector using Fast-link DNA ligase. After the ligation reaction, the solution was packaged into phage particles (MaxPlax Lambda Packaging Extracts) and transfected into TransforMax EPI300 *Escherichia coli (E. coli)*.

pCC2FOS vector contains a high copy origin of replication (*ori*V) as well as a chloramphenicol (CM) resistance gene. Under the regulated control of the arabinose-inducible promoter, the *trf*A gene product (supplied by TransforMax EPI300 cells) initiates replication from *ori*V and increases the copy number of the fosmid in the host. The host (EPI300^{TM}-T1R Phage T1-resistant *E. coli* plating strain) has the following properties:

[F– mcrA ∆(*mrr-hsd*RMS-*mcr*BC) (StrR) φ80dlacZ∆M15 ∆l*ac*X74 *rec*A1 *end*A1 *ara*D139 ∆(*ara, leu*)7697 *gal*U *gal*K λ– *rps*L *nup*G *trf*A *ton*A *dhfr*]

After packaging, titers of the packaged phage particles were estimated. As packed clones in foam had the highest concentration (84,025 CFU/mL (σ=5,414)), the foam metagenomic library was selected for further studies of antimicrobial screening and infected cells from influent (5,112 CFU/mL (σ=305)) and mixed liquor DNA (12,096 CFU/mL (σ=1,221)) were stored at -80°C. From the foam fosmid library, about 24,200 infected bacterial cells (29% of total) were plated on LB agar plates amended with chloramphenicol (CM) at a concentration of 12.5 µg/mL. After overnight incubation at 37°C, vector-positive colonies were picked by a QPix2 Robotic colony picker and inoculated in LB broth amended with 10% glycerol and CM (12.5 µg/mL) in 63 of 384-well microtiter plates. After overnight growth, the master library was copied (first copy) and both the master and copy libraries were stored at -80°C for further experiments. OD_{600} values of the 63 plates in the first copy was measured after the first freeze-thawing and the total number of viable clones was estimated to be 23,060.

In order to characterize the foam library, 10 fosmid clones were randomly selected and recombinant plasmids were extracted using EZ-10 Spin Column Plasmid DNA Minipreps Kit (BioBasic Company, Ontario, Canada). Plasmids were digested with BamHI restriction enzyme. In detail, 1 µL of BamHI (20,000 U/mL), 1 µL of NEB 3.1 buffer and ~400 ng of plasmid were mixed and an appropriate volume of nuclease-free sterile water was added to

obtain a final volume of 10 µL. The final solution was mixed and incubated at 37°C in a water bath for two hours. Resulting digests were analyzed by gel electrophoresis using 0.8% (wt/vol) ultra-pure agarose in 1 × TAE buffer stained with SYBR® safe DNA gel stain and visualized under UV light. Band patterns were analyzed to calculate the average size of the insert DNA in the foam fosmid library.

2.4.2.6 Functional Screening of the Foam Fosmid Library

The foam fosmid library was screened for resistance to carbenicillin (CARB) with the help of an undergraduate student (Doriane Loirat) in the Davies Laboratory. The library was first screened for resistance to CARB by growth in liquid cultures. Briefly, the copy library was replicated in LB broth by a QPix2 Robotic colony picker in 384-well microtiter plates supplemented with CM (12.5 µg/mL) and carbenicillin (CARB) (50 µg/mL). After overnight incubation at 37°C, OD_{600} measurements were collected and the total number of growing clones was determined. During the second screening, only potential carbenicillin-resistant ($CARB^r$) clones were tested for their growth in the presence of CARB (50 µg/mL) in LB agar media and growth-positive clones were selected for further analysis.

2.4.2.7 Identification of ARGs in $CARB^r$ Clones

$CARB^r$ clones in the foam fosmid library were characterized based on their patterns of digestion by BamHI restriction enzyme as well as their antimicrobial susceptibility profiles. The procedure of single digestion of fosmid DNA was described in the previous section and the methodology for antimicrobial susceptibility testing was presented in Section 2.4.1.1.

Fosmid DNA from $CARB^r$ clones was also transformed into *E. coli* DH10B electrocompetent cells to test if the observed resistance phenotypes were host independent. In detail, 1 µL of the fosmid DNA was mixed with 40 µL of DH10B competent cells and shocked in a 2 mm electroporation cuvette at 2.5 kV, 200 Ω and 25 µF using a BioRad Gene Pulser Xcell electroporation system. After the electric shock, 1 mL of pre-warmed LB broth was added to the bacterial suspension, mixed and then transferred into a sterile 10 mL glass tube and incubated at 37°C while shaking for 30 minutes. After the culture was centrifuged for two

minutes at 4000 g, 800 µL of the supernatant was discarded. Then, 100, 50 and 25 µL of the resuspended pellet were plated with glass beads on LB agar plates supplemented with CM (12.5 µg/mL) and CARB (50 µg/mL). Competency of *E. coli* dH10B cells was determined by transforming plasmid pUC19 carrying an ampicillin-resistant (AMP^r) marker into *E. coli* dH10B cells. The electroporation procedure was followed exactly as described with an exception of plating the final solution on LB agar plates supplemented with AMP (100 µg/mL).

The first step to identify ARGs in the EPI300:pF3.18 ($CARB^r$ clone) was the transposon mutagenesis procedure which was followed using the EZ-Tn5<KAN-2> Insertion kit protocol (Epicentre, Wisconsin, USA). Prior to constructing the Tn5 insertion library, fosmid DNA was digested using the Plasmid-Safe ATP-Dependent DNase kit (Epicentre, Wisconsin, USA) to remove the remaining linear chromosomal DNA. After the cleanup, an ethanol precipitation procedure was completed as previously described (Section 2.4.2.1).

Another requirement to construct the Tn5 insertion libraries was to prepare the *E. coli* EPI300 electrocompetent cells. In detail, fresh single colonies of the *E. coli* EPI300 strain were inoculated in 200 mL of 2YT broth. After overnight incubation at 37°C, the turbidity (OD_{600}) of cell suspension was adjusted to about 0.3 by dilution in 2YT medium. Then, the cells were incubated at 37°C under agitation and OD_{600} was measured every 20 minutes. As soon as OD_{600} reached 0.5-0.6, the cells were stored on ice for 15 minutes. The cells were then harvested in a 250 mL centrifuge bottle (previously cooled down on ice) and centrifuged at 3850 g for 10 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 200 mL of 2YT containing 10% glycerol. Centrifugation and resuspension of the pellet were repeated four more times until the resuspension volume was concentrated to 1 mL. Competent cells were then aliquoted in 1.5 mL microcentrifuge tubes and frozen immediately with liquid nitrogen (N) and stored at -80°C.

After *E. coli* EPI300 competent cells were prepared and the *in vitro* transposon insertion reaction was completed, electrotransformation was performed as previously described. Transformants were plated on two sets of LB agar plates, one set supplemented with CM (12.5 µg/mL) and one set amended with both CM (12.5 µg/mL) and KAN (50 µg/mL) to estimate the survival and transposition frequency. KAN-positive clones (KAN^r clones) were

then picked and screened to select for the loss of resistance phenotype. A control experiment monitored the viability of clones in LB medium supplemented with KAN (50 µg/mL) and CM (12.5 µg/mL).

As EZ-Tn5 Transposons contain unique primer-binding sites at each end, susceptible Tn5-inserted library clones were sequenced by NAPS (Vancouver, Canada) using KAN-2 forward primer (5′-ACCTACAACAAAGCTCTCATCAACC-3′) to identify the gene knockout. Trimming the sequence of Tn5 transposon and gene assembly were performed using macVector (v.13.0). Sequencing data were analyzed by performing a nucleotide BLAST search. In case primers were required to be designed, the primer designing tool at national center for biotechnology information (NCBI) website was applied.

3 RESULTS AND DISCUSSION

3.1 Release of SMX^r Bacteria in Membrane-treated Effluent and the Levels of the *sul*1 Gene in MEBPR Processes

In recent years, many studies have reported incidents of the release of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in treated-effluent irrigation water and effluent discharges to receiving surface water (Zhang et al., 2009; Li et al., 2009; Goñi-Urriza et al., 2000; Pruden et al., 2006; Reinthaler et al., 2003). As an example, Akiyama and Savin (2010) observed an increase in AR ratio in coliform bacteria downstream of a wastewater-polluted river in Arkansas, USA. Szczepanowski et al. (2009) detected a variety of plasmid-encoded resistance genes in ARB isolated from activated sludge and final effluent of a municipal WWTP in Germany. Auerbach and coworkers (2007) observed a larger diversity of tetracycline-resistant (tet^r) genes in wastewater treatment plant (WWTP) samples relative to the background natural lake water samples in Wisconsin, USA.

The relative abundances of ARB and ARGs in treated effluent generated by gravity separation of suspended solids in secondary settling tanks of conventional treatment processes have been frequently studied (Manaia et al., 2010; Lefkowitz and Duran, 2009; Gao et al., 2012; Sigala and Unc, 2012). However, the performance of advanced solids-liquid separation techniques in removal of ARB, as well as the diversity and resistance patterns of bacteria isolated from membrane-treated effluent, have been rarely monitored (Manaia et al., 2010; Fars et al., 2005; Zhang et al., 2015b).

The present section is focused on evaluating the performance of a membrane enhanced biological phosphorus removal (MEBPR) train operating at a 25 day SRT in controlling sulfamethoxazole (SMX) resistance. Performance assessment was done through three common approaches including (1) determination of the log removal of total heterotrophic bacteria and ARB to test for the efficiency of membrane filtration, (2) comparison of sulfamethoxazole-resistant (SMX^r) percentage in influent and effluent bacterial communities to examine if there was a selective increase in SMX resistance from influent to membrane effluent and, (3) comparison of the susceptibility profiles of 120 influent and treated effluent SMX^r bacteria against a set of five antibiotics from different classes to test if the percentage

of co-occurrence with SMX resistance increased in membrane-treated effluent relative to the influent. Data are presented in Sections 3.1.1 to 3.1.3.

To learn more about the diversity of cultivable heterotrophic dual-resistant and multi-drug resistant (MDR) bacterial communities in influent and treated effluent (which penetrate the ultrafiltration membrane permeate due to integrity breaches), all the 120 SMX^r bacterial isolates which conferred resistance to SMX and at least one more antibiotic were identified through 16S rRNA gene sequencing. That included a set of 16 (11 MH and 5 R2A) influent and 23 (14 MH and 9 R2A) effluent SMX^r bacterial strains (Section 3.1.4).

The last part of this section (3.1.5) focuses on investigating the role of very long solids retention time (SRT) operation on the levels of a SMX resistance gene in MEBPR processes. To satisfy this objective, the relative abundances of the *sul*1 gene (per 16S rRNA gene) in the redox zones of the UBC pilot plant parallel MEBPR trains operating at 25 and 60 day SRTs were compared. Meanwhile, comparing the concentration of the *sul*1 gene (gene copies per sample volume) in influent and membrane-treated effluent provided an opportunity for evaluating the efficiency of the membrane filtration in removal of the SMX^r gene. To satisfy the gap in knowledge regarding the unclear role of advanced treatment technologies in the attenuation or amplification of ARGs, the relative abundances of the *sul*1 gene copies (normalized to the 16S rRNA gene copies) were also compared between influent and mixed liquor.

Resistance to SMX was studied in this section. Sulfonamides such as SMX are antimicrobials which interfere with DNA synthesis by inhibiting the conversion of para-amino benzoic acid (PABA) to dihydropteroic acid (Gibreel and Sköld, 1999). The formation of dihydropteroic acid is catalyzed by dihydropteroate synthase (DHPS) in bacterial cells. Acting as competitive inhibitors of DHPS, SMX blocks a key step in the folate biosynthesis pathway (Huovinen et al., 1995). Resistance to SMX can be encoded by chromosomal mutation in the *fol*P gene and overproduction of the antimicrobial insensitive SMX^r DHPS enzymes (e.g. *sul*1 and *sul*2) (Balkhed, 2014).

Together with trimethoprim (TMP), SMX is one of the first-line antimicrobial agents for the treatment of urinary tract infections (BC Centre for Disease Control, 2010). This compound is one of the most commonly detected and studied antimicrobials in WWTPs and the

occurrence of SMX^r bacteria and *sul* genes on integron-harboring bacterial isolates in activated sludge has been frequently reported (Akiyama and Savin, 2010; Nagulapally et al., 2009; Ferreira da Silva et al., 2006; Munir et al., 2011; Ramsden et al., 2010; Munir and Xagoraraki, 2011; Galvin et al., 2010; Figueira et al., 2011; Ma et al., 2011; Fars et al., 2005; Okoh and Igbinosa, 2010; Mokracka et al., 2012).

3.1.1 Membrane Filtration Performance in Heterotrophic Bacterial Removal

In this study, the efficiency of a MEBPR train (SRT = 25 days) was evaluated by determination of the log removal of total cultivable heterotrophs and SMX^r heterotrophic bacteria through membrane filtration. To satisfy this objective, three influent and three membrane-treated effluent samples were collected on January 2014 in triplicate (biological replicates) and plated on MH and R2A media supplemented with and without SMX (50 µg/mL). After incubation, the number of growing colonies was counted. The procedure of sample collection was described in Section 2.2 and the methodologies of bacterial plating and enumeration were provided in Section 2.4.1.2.1. After cell counting, the removal of culturable bacteria on each medium (MH, R2A, MH+SMX, R2A+SMX) was determined using **Equation 2** in Section 2.4.1.2.3.

Total and SMX^r bacterial cell counts in influent and effluent samples are shown in **Figure 3.1**. As the standard deviation in plate counts of technical replicates was so small as to be negligible, an average of the three was used. Hence, each bar in **Figure 3.1** represents the mean colony forming units (CFUs)/mL (of biological replicates) ± standard error of the mean (SEM) of n=3 samples.

Comparing the influent and membrane-treated effluent colony counts, it was shown that membrane filtration achieved four and three log reductions of total heterotrophic and SMX^r bacteria with both media (MH and R2A), respectively (**Figure 3.1**). The high efficiency of the membrane filtration technique in removal of heterotrophic bacteria has been previously documented (Munir et al., 2011). As an example, Zhang and coworkers (2015b) compared the concentrations of bacteria in influent and effluent of three WWTPs in China and found that membrane bioreactors (MBRs) exhibited more than 20% increase in the removal percentage of total cultivable heterotrophic bacteria, compared to a secondary

sedimentation tank.

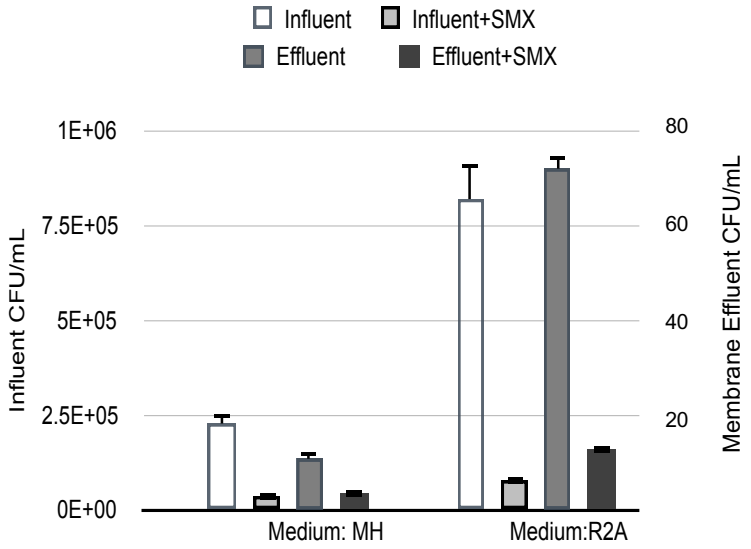


Figure 3.1 Comparison of the total heterotrophic and SMX^r bacterial cell counts between influent and membrane-treated effluent at SRT = 25 days (sampling: January 2014)

3.1.2 Comparison of SMX^r Percentage in Influent and Effluent Heterotrophic Bacterial Communities

The second aspect the 25 day SRT MEBPR train was studied was comparing the percentage of SMX^r cultivable heterotrophic bacteria in the influent and treated effluent. This was done by dividing the colony counts on SMX-supplemented agar plates (50 µg/mL) over the total number of bacteria growing on antimicrobial-free plates (MH or R2A) (Section 2.4.1.2.3). From **Figure 3.1**, the percentages of SMX^r MH and R2A heterotrophic bacteria were increased from 15.76% (σ=3.76%) to 34.45% (σ=6.37%) and 9.42% (σ=1.32%) to 18.27% (σ=1.22%) from influent to membrane effluent, respectively.

In summary, observations from Sections 3.1.1 and 3.1.2 implied that despite the overall high efficiency of membrane filtration in removal of total and SMX^r cultivable heterotrophic bacteria (four and three log reductions), a selective increase in the percentage of SMX^r ratio was observed from influent to membrane-treated effluent. Similar findings have been

reported by Zhang and coworkers (2009). While they observed about three orders of magnitude reduction in the population of total heterotrophs and *Acinetobacter spp.* from raw influent to effluent of a tertiary WWTP (in Ann Arbor, Michigan, USA), they observed selective increases in amoxicillin/clavulanic acid, chloramphenicol and rifampin resistance among *Acinetobacter spp.* isolated from effluent compared to those of the influent.

The increase of SMX resistance from influent to membrane-treated effluent could be attributed to the clonal selection of bacteria under the selective pressure of SMX in MEBPR processes (Ma et al., 2011). Horizontal transfer of sulfonamide resistance genes (*sul*1 and *sul*2) as well as variations in the composition of tested bacteria in influent and treated effluent could also be proposed as other possible causes of the increase in the percentage of SMX resistance in treated effluent. Parameters which can influence the role of wastewater treatment systems in elevation or reduction of ARB ratio are described in Section 3.2.

From **Figure 3.1**, it was also concluded that MH total heterotrophic bacterial cell counts were lower than those with R2A medium. Previous studies have shown that the medium composition, incubation time and temperature are important variables that affect the resulting plate counts (Gensberger et al., 2015; Vieira and Nahas, 2005). Although no literature studies were found which compared the cell counts of wastewater bacteria on MH and R2A media in a parallel study, it was expected that the colony counts in R2A medium, which allows detection of both fast- and slow-growing organisms (with low nutritional preferences), would be higher than with the MH medium. Previous work by Ćiric et al (2010) also reported significantly higher viable counts in R2A medium than those of the plate count agar.

In addition to the differences in the bacterial counts, the percentage of SMX resistance among heterotrophic bacteria cultured on MH and R2A media were also different. As MH and R2A media have major differences in nutrient levels (previously discussed in Section 2.4.1.2.1), it is expected that each medium supports the growth of different communities of heterotrophic bacteria. Hence, it is expected that the choice of medium creates differences in ARB percentage. The diversity of influent and membrane-treated effluent bacteria growing on MH and R2A media are studied further in Sections 3.1.4 and 3.2.5.

3.1.3 Comparison of the Antimicrobial Susceptibility Profiles of Influent and Effluent SMX^r Bacteria

The third approach to study the performance of the MEBPR system (SRT = 25 days) in dissemination of antimicrobial resistance (AR) was to compare the susceptibility patterns of SMX^r influent and treated effluent bacteria against a set of different antimicrobial agents. To satisfy this objective, 60 influent bacterial colonies (30 MH and 30 R2A bacteria) and 60 membrane-treated effluent bacterial colonies (30 MH and 30 R2A bacteria) were first randomly selected/isolated from the biological triplicate plates (SMX-supplemented) and purified by streaking and re-streaking on the initial fresh medium (MH or R2A). A total of 120 bacterial isolates was then investigated with respect to their antimicrobial susceptibilities to six antimicrobials (amoxicillin/clavulanic acid (AMC), chloramphenicol (CM), tetracycline (TET), trimethoprim (TMP), ciprofloxacin (CIP) and kanamycin (KAN)) by the disk diffusion method (methodology provided in Section 2.4.1.1).

After the susceptibility testing, the percentage of ARB in each community (influent and/ or effluent SMX^r bacterial isolates) was calculated by dividing the total number of bacterial isolates (regardless of the initial growth medium) which was resistant to an antimicrobial by the total number of tested strains in each sample (n=60) multiplied by 100. Data are shown in **Figure 3.2**. Detailed antimicrobial susceptibility testing results of MH and R2A influent and effluent SMX^r strains are provided in **Table A.1 (Appendix A). Figure A.1** presents the ratios of ARB in SMX^r tested strains in different media.

With respect to the origin of the sample (influent vs membrane effluent), SMX^r microorganisms isolated from treated effluent exhibited higher ARB percentages for TMP, TET, CM, KAN and AMC compared to those of the influent (**Figure 3.2**). However, the percentage of co-resistance of SMX with CIP in influent and effluent bacterial communities was not considerably different. While one reason for the elevation of ARB percentage in SMX^r bacteria could be the potential for acquisition of ARGs through horizontal gene transfer facilitated in the wastewater treatment process, it is highly likely that the variation in the tested bacterial isolates (imposed by media composition) in influent and membrane effluent caused differences in ARB ratios.

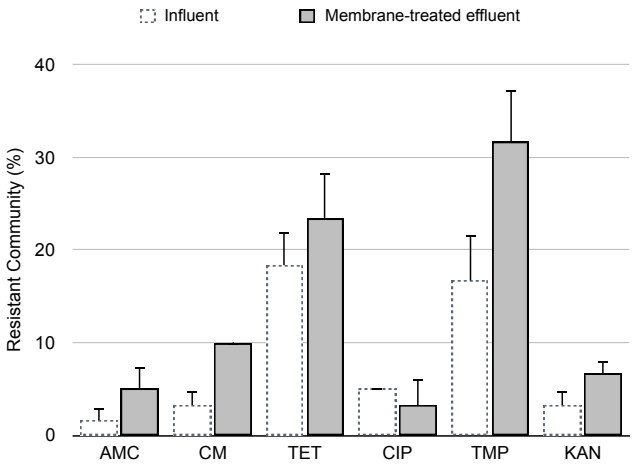


Figure 3.2 Comparison of the proportion of ARB between influent and treated effluent SMX^r tested strains (Sampling: January 2014, number of replicates for each sample = 3, number of tested isolates = 120)

Using the antimicrobial susceptibility data (presented in **Figure 3.2**), the percentage of the influent and membrane effluent SMX^r community which exhibited zero, one and multiple resistances in addition to SMX were also compared. This was done by grouping 60 influent and 60 membrane effluent SMX^r bacteria based on the number of resistances in each isolate. Data showed that out of the 60 influent SMX^r isolates, 26.7% were also resistant to one or several of the additional tested antibiotics (AMC, TET, TMP, CIP, KAN, CM); the corresponding proportion in effluent bacteria was 38.3%. In detail, 11.7% of influent bacteria showed a single co-resistance (SMX and one antimicrobial), 8.3% and 6.7% had double (SMX and two antimicrobials) and multiple co-resistances, respectively. In effluent bacteria, however, the percentage of single co-resistance increased to 13.3% and the total percentages of double and multiple co-resistances increased to 25%.

Although membrane filtration appears to be a promising approach to achieve superior ARB removal per unit volume (presented in Section 3.1.1), observations in Sections 3.1.2 and 3.1.3 showed that not only the ratio of SMX resistance in cultivable heterotrophic bacteria considerably increased from influent to membrane-treated effluent, but also the population of

SMX^r bacteria with single, double or multiple resistances exhibited an elevation in the treated effluent compared to that of the influent.

Current observations support the necessity for the application of supplementary techniques to ensure the quality of membrane effluent prior to discharge or reuse. In this respect, including microbiological techniques, such as bacterial plating (which could be used for determination of ARB ratios in membrane-treated effluent), to the list of routine physiochemical water quality parameters, seems to be necessary.

3.1.4 Identification of SMX^r Dual-resistant and MDR Bacteria in Influent and Membrane-treated Effluent

Due to the nominal pore size (0.04 μm) of the UBC pilot plant hollow fiber membrane filtration modules, size exclusion could achieve the total removal of bacteria if the membranes were fully intact. However, observations from Section 3.1.1 documented the presence of SMX^r heterotrophic bacteria ((3.87 CFU/mL in MH (σ=0.46) and 13.27 CFU/mL in R2A medium (σ=0.74)) in the permeate of the MEBPR system operating at a 25 day SRT. This observation is in line with previous studies which have reported the occurrence of ARB and ARGs in effluent from MBRs (Munir et al., 2011; Xia et al., 2012). It was previously discussed (Section 1.4) that the membrane integrity breaching frequency is highly affected by parameters such as membrane aging in which the potential of pore expansion due to periodic exposure to chemical agents, chemical degradation of membranes as well as mechanical damage, is maximized.

In this section, all of the dual-resistant and MDR SMX^r bacteria (n = 16 in influent and n = 23 in permeate), from the 120 previously tested bacterial isolates), were identified by 16S rRNA gene sequencing to obtain basic information on the diversity of the antibiotic resistant cultivable heterotrophic bacteria in influent and membrane permeate. As identified bacterial strains (n=39) had also been grouped based on their initial isolating medium, the diversities of heterotrophic ARB growing on each medium (MH or R2A) were also studied.

The methodologies for preparation of crude bacterial cell lysates as well as PCR amplification and sequencing procedures were described in Sections 2.4.2.1 and 2.4.2.2.

Distribution of dual-resistant and MDR SMX^r influent and membrane effluent bacteria is shown in **Figure 3.3**. Phylogenetic trees as well as antimicrobial susceptibility results corresponding to identified influent and effluent bacteria are presented in **Figures A.2** and **A.3** (**Appendix A**). Phylogenetic trees were built with the sequences of the V3 region of the 16S rRNA gene. **Figures A.4** and **A.5** (**Appendix A**) compared the composition of identified influent and effluent bacteria at the order and class levels.

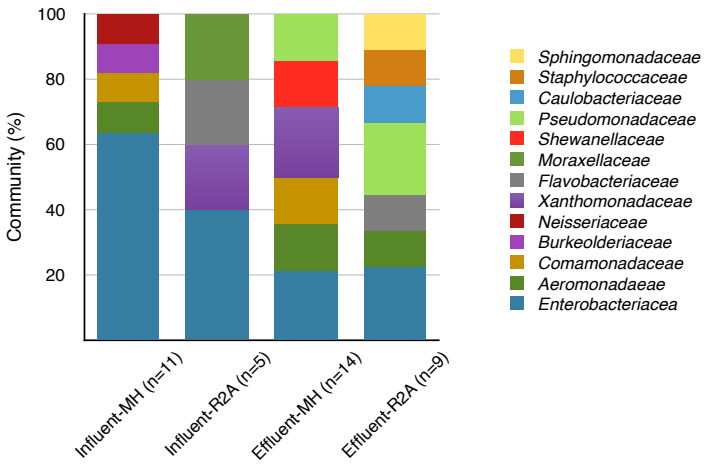


Figure 3.3 Composition of influent and membrane effluent SMX^r 16S-sequenced bacterial isolates at the family level (Sampling: January 2014)

From **Figure 3.3**, it can be observed that the most abundant group of dual-resistant and MDR bacteria in the influent, representing between 40% to 63% of the total community of MH and R2A bacteria, was from the family *Enterobacteriaceae*. Along with the enteric bacteria, bacterial isolates from the families *Xanthomonadaceae* (relative abundance: 21% in MH medium) and *Pseudomonadaceae* (relative abundance: 22% in R2A medium) were also abundant among MH and R2A SMX^r dual-resistant and MDR effluent bacteria, respectively. Commonly in both influent and effluent environments, SMX^r bacteria were detected among bacteria affiliated with the families *Enterobacteriaceae, Aeromonadaeae*, *Comamonadaceae*, *Xanthomonadaceae* and *Flavobacteriaceae*. However, bacteria from *Burkholderiaceae*, *Neisseriaceae* and *Moraxellaceae* families were only isolated from

influent and bacteria affiliated with the families S*hewanellaceae*, *Pseudomonadaceae*, *Caulobacteraceae*, *Staphylococcaceae* and *Sphingomonadaceae* were only detected in the membrane effluent. It should be noted that, due to the limitation in the number of identified strains (16 influent and 23 membrane-treated effluent bacterial isolates), comparison of the bacterial community structure of cultivable heterotrophic SMX^r bacteria in the two environments was not performed.

As previously described, the scope of most of the previously published AR studies on treated effluent from WWTPs is limited to documenting the resistance profiles of single population subsets of effluent bacteria such as indicators of faecal contamination or other specific pathogenic strains (Al-Bahry et al., 2009; Okoh and Igbinosa, 2010; Ma et al., 2011; Lefkowitz and Duran, 2009; Akiyama and Savin., 2010; Zhang et al., 2009). Observations from this section of the present study showed that the community of dual-resistant and MDR SMX^r bacteria in membrane effluent was not limited to indicators of faecal contamination such as *E. coli* (**Figure 3.3**). This highlights the point that, in order to make more accurate estimations of ARB release rates in membrane effluent, ARB ratios need to be evaluated for the total heterotrophic bacteria.

In general, the majority of SMX^r bacteria isolated from either MH or R2A medium belonged to the class *Gamma-proteobacteria* (>73%). In addition, all strains from the class *Beta-proteobacteria* (18.7% of the influent population and 8.7% of the effluent community) were isolated on MH medium whereas all bacteria affiliated with the classes *Bacilli*, *Alpha-proteobacteria* and *Flavobacteria* were isolated on R2A medium. Considering the differences in the formulation of MH and R2A media (nutrient-high vs nutrient-low), preferential growth of different diversities of heterotrophs on each medium is expected. Allen and coworkers (2004) summarized observations in the literature on heterotrophic plate counts and populations of a variety of heterotrophic media including R2A, nutrient agar and standard plate count agar. While they classified high-nutrient media to be suitable for the enumeration of bacteria from animals and humans, they proposed low-nutrient media to isolate bacteria with a water-based lifestyle.

Monitoring the antimicrobial susceptibility profiles of 39 identified bacterial isolates showed that most of the bacterial genera with a sufficient sample size (five or more strains) had

similar resistance phenotypes (**Figures A.2** and **A.3**). As an example, the majority of influent SMX^r bacteria from the family *Enterobacteriaceae* was resistant to TMP (71%) and TET (100%) (**Figure A.2**). In addition, simultaneous co-resistance of SMX with TMP and TET was a common finding in all effluent *Escherichia, Stenotrophomonas* and *Pseudomonas* strains (**Figure A.3**). In total, five of the seven *E. coli* strains in the influent (71%), and all of the *E. coli* strains in the effluent, were resistant to at least two of the five tested antibiotics.

Table 3.1 provides a list of identified bacterial isolates in this section at the genus level. In most of the 16S-sequenced genera of effluent bacteria, there is at least a known species capable of combined heterotrophic nitrification and aerobic denitrification, P reduction or organic removal which emphasizes the potential for growth and reproduction of ARB in the wastewater treatment environment (**Table 3.1**). Knowing that these dual-resistant and MDR SMX^r bacteria have at least one well-recognized pathogenic species in their genera, calls for the need for routine and continuous microbial water quality assessment of MBR effluent, to control the emission of ARB more specifically in cases where membrane filtration is the final stage, prior to further application or discharge.

Table 3.1 List of influent and membrane-treated effluent bacterial genera identified by 16S rRNA gene sequencing (presented in Section 3.1.4)

Bacterial Genus	No. of Bacterial Isolates				Example of Pathogenic Strain	Example of Studied Species	Reference	Studied Activity
	Influent		Effluent					
	MH	R2A	MH	R2A				
Acidovorax	-	-	1	-	*Acidovorax valerianellae* (plant pathogen)	*Acidovorax caeni*	Heylen et al., 2008	denitrification
Acinetobacter	-	1	-	-	*Acinetobacter baumannii*	*Acinetobacter sp. HA2*	Yao et al., 2013	nitrification/ denitrification
						Acinetobacter calcoaceticus	Sidat et al., 1999	P reduction
Aeromonas	1	-	2	1	*Aeromonas hydrophila*	*Aeromonas sp. HN-02*	Chen et al., 2014	nitrification/ denitrification
						Aeromonas hydrophila	Naili et al., 2015a	P reduction
Burkholderia	1	-	-	-	*Burkholderia cepacia*	*Burkholderia cepacia*	Ajao et al., 2013	organic degradation
Chryseobacterium	-	-	-	1	*Chryseobacterium meningosepticum*	*Chryseobacterium sp. R31*	Kundu et al., 2014	nitrification/ denitrification
Citrobacter	-	1	-	-	*Citrobacter freundii*	*Citrobacter diversus*	Huang and Tseng, 2001	denitrification
Cloacibacterium	-	1	-	-	-	*Cloacibacterium normanense*	Nouha et al., 2016	heavy metal removal
Comamonas	1	-	1	-	*Comamonas testosteroni*	*Commonas sp. SGLY2*	Patureau et al., 1997	nitrification/ denitrification
Enterobacter	1	-	-	-	*Enterobacter cloacae*	*Enterobacter cloacae*	Naili et al., 2015b	denitrification
						Enterobacter sp. KLW-2	Krishnaswamy et al., 2011	P reduction
Escherichia	6	1	2	2	*E. coli*	*E. coli K12*	Kulaev et al., 2004	P reduction
						E. coli FDY10	Mazzucotelli et al., 2014	organic degradation
Pseudomonas	-	-	2	2	*Pseudomonas aeruginosa*	*Pseudomonas luteola*	Naili et al., 2015b	denitrification
						Pseudomonas sp. YLW-7	Krishnaswamy et al., 2011	P reduction
Phenylobacterium	-	-	-	1	-	-	-	-
Shewanella	-	-	2	-	*Shewanella algae*	*Shewanella decolorationis*	Xu et al., 2005	dye decolorization
Sphingopyxis	-	-	-	1	*Sphingomonas paucimobilis*	-	-	-
Staphylococcus	-	-	-	1	*Staphylococcus aureus*	-	-	-
Stenotrophomonas	-	1	4	-	*Stenotrophomonas maltophilia*	*Stenotrophomonas sp. DIV102*	Mazzucotelli et al., 2014	organic degradation
Uruburuella	1	-	-	-	-	*Uruburuella suis*	Vela et al., 2005	denitrification

3.1.5 Relative Abundances of the *sul*1 Gene in Influent and MEBPR Systems

The application of PCR and real-time quantitative PCR (qPCR) approaches in wastewater treatment systems has increased our knowledge of the structure and population dynamics of

wastewater microbial communities such as those that support polyphosphate accumulation, fermentation, nitrification and denitrification activities (Aoi et al., 2005; Bahadoorsingh, 2010; Lawson, 2014).

Many studies in recent years have also used PCR-based techniques to detect and quantify a diversity of AR determinants in influent, activated sludge and treated effluent of WWTPs (Gao et al., 2012; LaPara et al., 2011; Lachmayr et al., 2009). In most of these studies, the elevation or reduction of particular ARGs from influent to treated effluent has been evaluated. While the scope of qPCR assays is limited to the quantification of previously described ARGs, these techniques overcome the error induced by cultivation approaches and provide opportunities for the analysis of the true diversity of wastewater bacterial communities (Kim et al., 2013).

This section addresses the objective of the project regarding the role of long SRT operations on the relative abundance of a sulfonamide resistance gene in MEBPR processes. Prior to qPCR assay testing, qualitative PCR reactions were performed and the presence of two sulfonamide resistance genes (*sul*1 and *sul*2) was confirmed in total genomic DNA of influent and membrane-treated effluent samples collected on January 2014. The *sul*1 gene was then selected to be quantified by real-time qPCR assays. As previously noted, resistance to SMX can be acquired through horizontal transfer of genes which encode alternative forms of the DHPS enzyme. Often located on mobile genetic elements (MGEs), the *sul*1 gene is part of the 3′ conserved segment of class 1 integrons and hence, the occurrence of this gene is correlated to other ARGs in this class (Antunes et al., 2005). The presence of the *sul*1 gene in genomic and plasmid DNA of wastewater samples have been previously documented in the literature (Xia et al., 2012; Gao et al., 2012; Ma et al., 2011; Suhartono, 2016; Tennstedt et al., 2003).

Quantification of the *sul*1 gene in parallel MEBPR processes in the present project was performed on samples collected from influent, mixed liquor (anaerobic, anoxic and aerobic redox zones) as well as membrane-treated effluent of the UBC pilot plant dual trains operating at 25 and 60 day SRTs over a period of 13 months (sampling events: January, June and December 2014 as well as January and February 2015). The sampling plan, the DNA extraction procedure, list of primers as well as a detailed description of the gene

quantification procedure were provided in Sections 2.2 and 2.4.2.1 to 2.4.2.3. The standard curve, the qPCR amplification curve as well as the melt peak curve obtained from quantification of the *sul*1 gene are shown in **Figures A.6**, **A.7** and **A.8 (Appendix A)**.

The relative abundances of the *sul*1 gene in the parallel MEBPR processes are shown in **Figure 3.4**. It should be noted that the number of copies of the *sul*1 gene in each sample was normalized to the bacterial 16S rRNA gene copies as a surrogate measure of the total bacterial abundance. Box plots represent the distribution of data by means of a five-number summary, the minimum, the lower quartile (25th percentile), the median (50th percentile), the upper quartile (75th percentile), and the maximum. Average values of the normalized *sul*1 levels at different sampling points and times are shown in **Table A.2** (**Appendix A**).

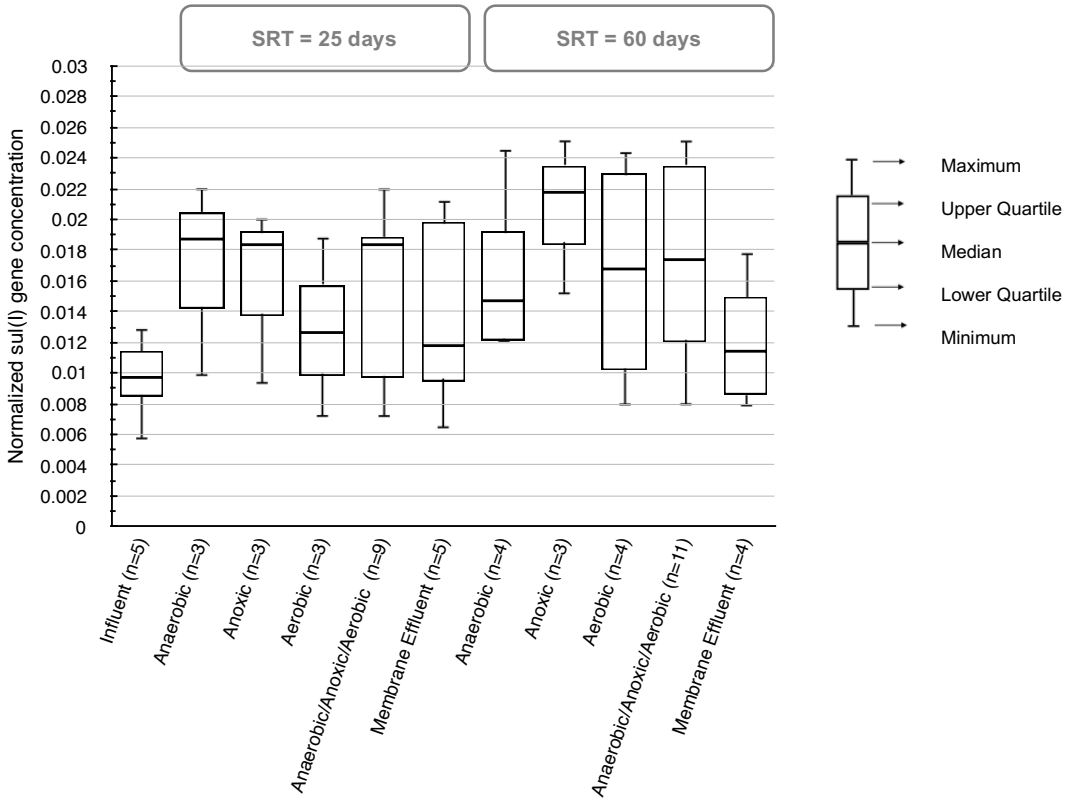


Figure 3.4 Comparison of the *sul*1 gene levels (normalized to 16S rRNA gene) in influent and MEBPR environments

From **Figure 3.4**, it can be seen that, compared to influent, the normalized levels of the *sul*1 gene in membrane effluents were not statistically significantly different ($P > 0.05$) at either 25 or 60 days of sludge age. As no significant difference ($P > 0.05$) was observable in the average values of the normalized concentrations of the *sul*1 gene between the anaerobic, anoxic, and aerobic mixed liquors of the parallel MEBPR trains at either 25 or 60 day SRTs, data corresponding to the *sul*1 gene levels in redox zones were pooled, as shown in **Figure 3.4**.

Comparing the mixed liquor and influent, the relative *sul*1 gene levels significantly ($P \leq 0.05$) increased from influent to mixed liquor at 25 and 60 day SRTs. However, no statistically significant ($P > 0.05$) difference was observed in the normalized *sul*1 gene levels of mixed liquor at the 60 day SRT train compared to that of the system operating at SRT = 25 days.

Different parameters such as the presence of SMX at sub-inhibitory levels and higher frequency of the *sul*1 gene transfer at 25 and 60 day SRT operations could be proposed as two potential causes of the elevated relative abundances of the *sul*1 gene in mixed liquors compared to those of the influent. However, as the concentration of SMX was not monitored in influent and parallel MEBPR processes in the present project, examining if there was a correlation between SMX levels and their degradation products to the normalized *sul*1 levels was not feasible. Although measuring the abundance of a specific gene determinant cannot be used to estimate the levels of other ARGs conferring resistance even to the same antimicrobial, determination of the frequency of transfer of individual ARGs could be proposed as a useful approach to provide an understanding of the potential of genetic exchange when SRT is extended.

While data from **Figure 3.4** show that the relative abundances of the *sul*1 gene (normalized to 16S rRNA gene or total bacterial community) in influent and membrane-treated effluents at 25 and 60 day SRTs were not significantly different ($P > 0.05$), the absolute concentrations of the *sul*1 gene in influent (normalized to the original sample volume) were found to be significantly higher than those of the treated effluents (**Figure 3.5**). In detail, membrane filtration achieved about three log reductions ($4.9 \times 10^{5} \pm 1.6 \times 10^{5}$ *sul*1 copies/mL in influent vs $5 \times 10^{2} \pm 2 \times 10^{2}$ in effluent) of the *sul*1 gene in the parallel wastewater treatment processes. The reduction of ARG concentrations from influent to effluent has also

been previously observed by Walston (2013) who showed that *sul*1, *sul*2, *dfr*(A1), *tet*(W) and *van*(A) concentrations declined three to four orders of magnitude from influent to secondary effluent at two municipal WWTPs operating at 19 and 45 day SRTs in Arizona, USA. Consistent with observations in **Figure 3.5**, the ratio of the *sul*1 gene release rate in membrane effluent to the inflow rate was negligible (0.0015 ± 0.0012 at SRT = 25 days and 0.0009 ± 0.0004 at SRT = 60 days) as calculated using **Equation 10** in Section 2.4.1.2.3.

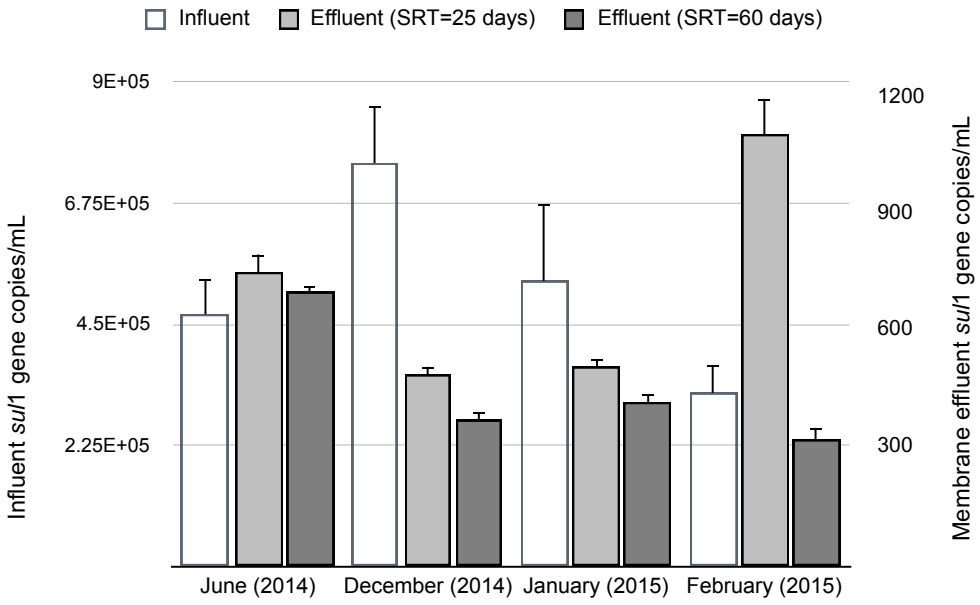


Figure 3.5 Comparison of the influent and membrane-treated effluent *sul*1 gene copies/mL

Comparing observations presented in **Figures 3.4** and **3.5** shows that normalizing resistance determinants to 16S rRNA gene copies and/ or sample volume could influence the way the performance of solids-liquid separation techniques is evaluated. In this respect, monitoring the relative abundance of ARGs normalized to 16S rRNA gene copies in mixed liquor could be used as a more efficient tool to evaluate the potential of horizontal gene transfer (HGT) in wastewater treatment systems. The reason is that elevation in the absolute concentration of an ARG (gene copy per unit volume) in mixed liquor could be due to the elevated abundance of bacterial communities per unit sample volume compared to that of

the influent. Xia and coworkers (2012) observed an elevation in the copies of bacterial 16S rRNA gene per unit volume when the levels of suspended solids in mixed liquor increased. Hence, it is expected that, at long SRT conditions, where generally higher levels of suspended solids are maintained, higher total bacterial 16S rRNA gene copies are present in mixed liquor per unit volume relative to those of the influent. Hence, under similar normalized concentrations of an ARG (gene copies/16S rRNA copies) between influent and mixed liquor, the copies of the resistance determinant per unit volume will be higher in mixed liquor, which could interfere with interpretation of data in terms of the adverse effects of wastewater treatment process in controlling AR.

A summary of conclusions in Section 3.1 includes the following.

- In the present study, the performance of the MEBPR system operating at 25 day SRT was evaluated through (1) determination of the log removal of cultivable heterotrophic and SMX^r bacteria, (2) comparison of the ratio of SMX resistance in influent and membrane-treated effluent bacterial communities and (3) comparison of the susceptibility profiles of SMX^r cultivable heterotrophic strains in influent and treated effluent bacteria. The results indicated that while membrane filtration achieved more than three log reductions of total cultivable heterotrophic and SMX^r bacteria, operating the MEBPR system at SRT = 25 days increased the proportion of SMX resistance as well as the percentages of TMP^r, TET^r, CM^r, KAN^r and AMC^R in treated effluent SMX^r tested bacteria compared to those of the influent. This implies that operation of the MEBPR system at SRT = 25 days could enhance the potential for gene transfer and production of ARB among heterotrophic bacterial communities.
- Although the studied cultivable MH and R2A heterotrophic bacterial communities in treated effluent are not representative of the true diversity of bacterial composition in this environment, 16S rRNA gene sequencing of bacterial isolates provided a base knowledge of the diversity of cultivable heterotrophs in the permeate. In the present section, bacteria affiliated with the families *Sphingomonadaceae, Enterobacteriaceae, Pseudomonadaceae*, *Xanthomonadaceae*, *Aeromonadaeae*, *Comamonadaceae*, *Shewanellaceae, Caulobacteraceae*, *Flavobacteriaceae* and *Staphylococcaceae* were detected in the membrane-treated effluent of the 25 day

SRT train.

- Quantification of the *sul*1 gene in influent and parallel MEBPR trains showed that the elevation of the normalized concentrations of the *sul*1 gene copies (to 16S rRNA gene copies) in mixed liquor (25 and 60 day SRTs) relative to the influent was statiscially significant ($P \leq 0.05$). Consistent with observations on the removal of total heterotrophic and SMX^r bacteria, membrane filtration exhibited about three log reductions of the *sul*1 gene (per unit volume), in parallel MEBPR systems.

3.2 The Role of Long SRT Operations in Release of ARB from MEBPR Processes

Concerns regarding the leakage of antibiotics and antibiotic resistant bacteria (ARB) from WWTPs have led to the suggestion that wastewater treatment processes are incubators for the generation of resistant bacteria (Reinthaler et al., 2003; Zhang et al., 2009; Figueira et al., 2011). It has been shown that wastewater treatment system operating parameters such as SRT have critical effects on microbial diversity, the efficiencies of organic carbon oxidation as well as removal of nitrogen (N) and micropollutants in an activated sludge process (Clara et al., 2005; Kim et al., 2005; Kim et al., 2011; Liu and Wang, 2014). However, it is not known whether very long SRT operations may affect the distribution of ARB in WWTP microbial populations.

The present section is aimed to examine the effects of very long solids retention time (SRT) operations on the potential patterns of release of ARB from membrane enhanced biological phosphorus removal (MEBPR) processes. Release of ARB from wastewater treatment systems generally occurs through two major pathways, effluent discharge and waste biosolids. Observations from Section 3.1 showed that while a considerable reduction was observed in colony counts of total heterotrophic and sulfamethoxazole-resistant (SMX^r) bacteria (represented by comparison of colony forming units (CFUs)/mL) from influent to MEBPR effluent at the 25 day SRT, the membrane permeate pathway is of importance due to the increased percentage of ARB as well as multi-drug resistant (MDR) bacteria in membrane permeate relative to the influent. The occurrence of ARB and antibiotic resistance genes (ARGs) in biosolids-amended soils has also been frequently reported (Munir et al., 2011; Munir and Xagoraraki, 2011) which turns the waste solids pathway into a

potential candidate to study ARB release rates.

In the UBC pilot plant MEBPR systems, SRT was controlled through the discharge of the excess suspended solids in either foam or aerobic zone mixed liquor. As an initial step, all the foam formed over a period of 24 hours was regularly harvested and disposed and that was followed by aerobic zone solids wasting, if required. Hence, with particular focus on membrane-treated effluent, waste aerobic mixed liquor and waste foam, the performance of parallel MEBPR systems operating at 25 and 60 day SRTs was compared by evaluating the following parameters:

1. The percentage of ARB in total heterotrophic bacteria in influent, mixed liquor, foam and membrane-treated effluent (Section 3.2.1),
2. MDR population of total heterotrophs in influent, mixed liquor, foam and membrane-treated effluent (Section 3.2.2),
3. Log removal of ARB through membrane filtration (Section 3.2.3), and
4. Rate of ARB release in waste mixed liquor, foam and membrane effluent (Section 3.2.4).

In order to determine the above-mentioned parameters, bacterial colonies isolated from samples of influent, anoxic mixed liquor, foam and treated effluent of the MEBPR processes (SRT = 25 and 60 days) on December 2014, January and February 2015 were screened for resistance to sulfamethoxazole (SMX) (50 µg/mL), trimethoprim (TMP) (5 µg/mL), amoxicillin (AMX) (32 µg/mL), tetracycline (TET) (10 µg/mL) and ciprofloxacin (CIP) (5 µg/mL) by growth in liquid cultures. Screening was done on the isolating media MH and R2A to support a diverse group of cultivable heterotrophic bacteria. Details of the procedure for monitoring the ARB growth in liquid media were provided in Section 2.4.1.2.2. In total, more than 4,200 MH- and 3,100 R2A-grown heterotrophic bacteria from seven sampling points were screened for resistance to ensure broad representation of antibiotic resistant bacteria (ARB).

In addition, this section was also intended to provide additional information on the diversity of cultivable ARB in membrane-treated effluent. To address this research question, the lactose fermenting proportions of 96 MH and 96 R2A treated effluent strains were compared and a set of 33 MH and R2A effluent bacterial isolates were identified by 16S rRNA gene sequencing at the genus level. The resulting data are presented in Section 3.2.5.

3.2.1 Percentage of ARB in Influent and WWTP Environments

ARB ratios of cultivable heterotrophic bacteria to antimicrobials were calculated by dividing the total number of organisms with positive growth in antimicrobial-supplemented media by the total number of viable organisms in antimicrobial-free media (Section 2.4.1.2.3). **Figures 3.6** and **3.7** present the ARB proportions of bacteria in influent and the parallel MEBPR processes. Each bar represents the mean percentage of ARB ± (standard error of the mean (SEM) of n=3 samples collected on December 2014, January and February 2015). Detailed ARB ratios obtained at different sampling times and points are provided in **Table B.1**.

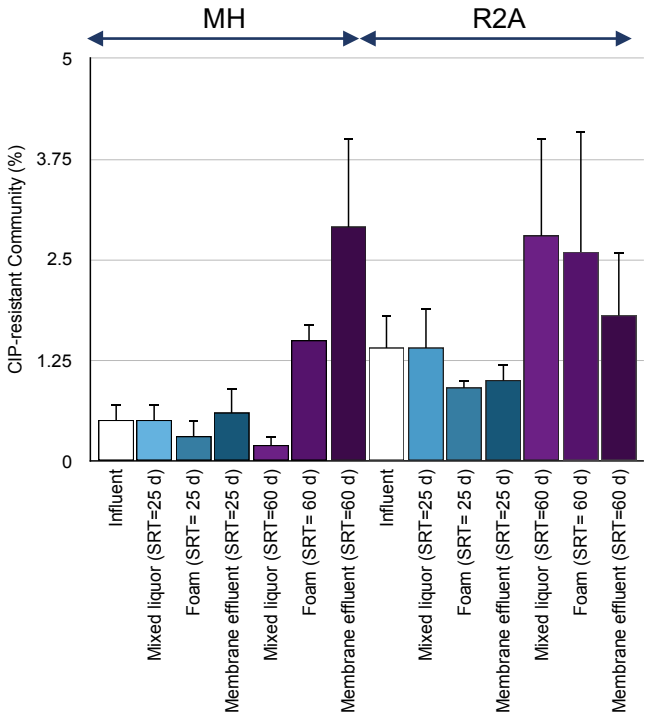


Figure 3.6 Percentage of CIP^r bacteria in influent and MEBPR environments (Sampling: December 2014, January and February 2015, total number of tested bacteria: 4,219 MH and 3,156 R2A isolates)

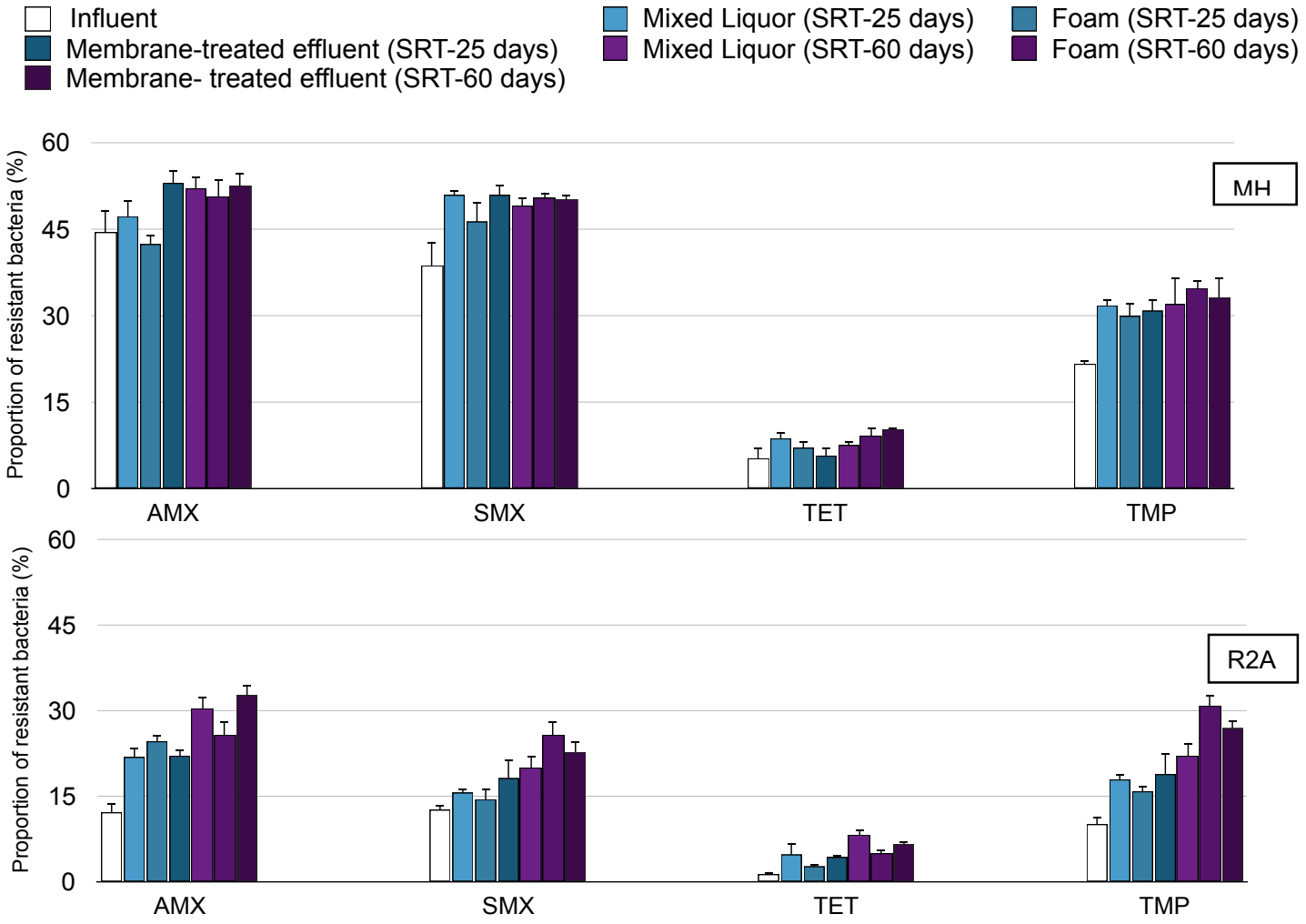


Figure 3.7 Percentage of ARB in influent and MEBPR environments (Sampling: December 2014, January and February 2015, total number of tested bacteria: 4,219 MH and 3,156 R2A isolates)

Tables B.2 to **B.4** (**Appendix B**) summarize the results of the t-based hypothesis tests to compare if the difference between two mean values of ARB ratios was statistically significant at 5% and 10% significance levels.

From **Figures 3.6** and **3.7**, it can be observed that AMX was associated with the highest abundance of resistant bacteria (AMX^r ratio: 48.8% ± 4.2% in MH and 24.1% ± 6.7% in R2A populations) and CIP was associated with the lowest abundance of resistant bacteria (AMX^r ratio: 0.93% ± 0.97% in MH and 1.7% ± 0.75% in R2A populations) in influent and MEBPR environments, including mixed liquor, foam and treated effluent. In detail, in the influent MH community, no significant differences ($P > 0.05$) were observed in the percentages of AMX^r and SMX^r bacteria. In the influent R2A population, no significant differences ($P > 0.05$) were observed in resistance ratios of AMX, SMX and TMP in cultivable heterotrophic bacteria.

As presented in Section 2.4.1.2.2 (**Table 2.5**), the target antimicrobials applied in the present project have different modes of action and are subject to different mechanisms of resistance. Since the taxnomomic composition of isolated bacteria and the genes conferring resistance to the tested antimicrobials were not known, understanding the predominant mechanism of resistance and if ARGs were located on the chromosome or on mobile genetic elemenets (MGEs) such as plasmids, integrons and insertion sequences (ISs) was not possible.

However, observations from **Figures 3.6** and **3.7** highlight the point that, the percentages of resistance to AMX, SMX, and TMP among heterotrophic bacteria in the influent and MEBPR compartments of the UBC pilot plant were generally higher relative to the CIP^r and TET^r ratios. The increased percentages of AMX^r, SMX^r and TMP^r bacteria could be due to many factors; the composition of bacterial communities in each environment (e.g. influent, mixed liquor, foam, membrane effluent), the composition of isolating media (MH and R2A) which influences the diverstity of cultivable heterotrophic bacterial communities isolated from each environment, the frequency of prescription, usage and disposal of antimicrobials on the UBC campus, the concentration of antibiotics in influent and their fate and frequency of gene transfer through the wastewater treatment processes.

In general, co-trimoxazole (SMX and TMP combination) is of the most commonly prescribed antimicrobials for the treatment of lower respiratory and urinary tract infections (Mehnert-Kay, 2005). In addition, genes conferring resistance to SMX, TMP and AMX have been

frequently detected in plasmids isolated from bacteria in hospital water and raw sewage (Chonova et al., 2016; Kümmerer and Henninger, 2003; Nagulapally et al., 2009; Fars et al., 2005). Due to the potential for the existence of these antimicrobials at sub-inhibitory levels in WWTPs and the presence of some corresponding ARGs on mobile genetic elements (MGEs), detection of large populations (42.3%-52.9% AMX^r ratio in MH bacteria and 12.1%-32.6% AMX^r ratio in R2A bacteria, 38.5%-50.8% SMX^r ratio in MH bacteria and 12.5%-25.7% SMX^r ratio in R2A bacteria, 21.4%-34.6% TMP^r ratio in MH bacteria and 9.9%-30.8% TMP^r ratio in R2A bacteria) of wastewater bacteria resistant to these antimicrobials is expected.

Resistance to CIP and TET in bacterial isolates from activated sludge and effluent of WWTPs has also been previously documented (Santoro et al., 2015; Rijal et al., 2009; Kim et al., 2007a; Duong et al., 2008; Fars et al., 2005). Consistent with observations from the present section (**Figure 3.6** and **3.7**), previous work by Gao et al. (2012) reported higher ratios of SMX resistance compared to TET resistance in raw influent total heterotrophic bacteria in a WWTP in East Lansing MI, USA. Manaia and coworkers (2010) observed that proportions of CIP^r heterotrophic bacteria (isolated from plate count agar medium) in raw influent and treated effluent of five WWTPs in Portugal ranged from 1.7% to 4.4% which are similar to the ratios of CIP resistance as those determined in the present study (0.2% to 2.9% in MH population and 0.9% to 2.8% in R2A heterotrophic population) (**Figure 3.6**).

Comparing the ARB proportions between influent and mixed liquor, SMX and TMP showed statistically significant increases in the dual treatment trains in both MH and R2A communities through the wastewater treatment processes (**Figure 3.7**, **Table B.1**). In detail, the ratios of SMX and TMP resistance in the MH population significantly ($P \leq 0.05$) increased (38.5% ± 7.1% in influent vs 50.8% ± 1.4% in mixed liquor, 21.4% ± 1.2% in influent vs 31.7% ± 1.9% in mixed liquor for SMX and TMP, respectively) through the treatment process at a 25 day SRT train. In the MEBPR system at a 60 day SRT, resistance ratios of SMX and TMP increased in the mixed liquor MH community (38.5% ± 7.1% in influent vs 48.9% ± 2.7% in mixed liquor, 21.4% ± 1.2% in influent vs 31.8% ± 8.2% in mixed liquor for SMX and TMP, respectively) relative to those of the influent at 10% significance level. In the R2A populations, ratios of SMX, TMP and AMX resistance in mixed liquor exhibited quantifiable elevations (3%, 7.8% and 9.6% for SMX, TMP and AMX, respectively)

from those of the influent at a 25 day SRT. Except for CIP, statistically significant ($P \leq 0.05$) increases were also observed in resistance ratios of the other antimicrobials in the R2A mixed liquor microbial community at a 60 day SRT compared to those of the influent.

Comparing the percentage of ARB in influent and treated effluent heterotrophic bacterial communities, statistically significant increases were also observed in ratios of SMX ($P \leq 0.1$) and TMP ($P \leq 0.05$) resistance in parallel trains in the MH populations from influent. However, no significant differences were found in CIP^r and AMX^r proportions of influent and membrane-treated effluent bacteria in the 25 and 60 day SRT trains (**Figures 3.6** and **3.7**). Ratios of TET resistance also increased in both MH and R2A populations from influent to MEBPR effluent at the 60 day SRT train (5.1% ± 3.1% in influent vs 10.1% ± 0.8% in effluent in MH population and 1.1% ± 1.0% in influent vs 6.5% ± 0.6% in effluent in R2A population) (**Figure 3.7**, **Table B.1**). It should also be noted that SMX^r, AMX^r, TET^r and TMP^r ratios of the R2A community also exhibited significant elevations ($P \leq 0.05$) in the treated effluent of the 60 day SRT train compared to those of the influent.

Statistical analysis of data also showed that the resistance ratios of mixed liquor and effluent bacteria at a 25 day SRT in MH and R2A populations were not significantly different ($P > 0.05$). In the 60 day SRT train, the ratio of TET resistance exhibited a significant increase in the MH mixed liquor community (7.5% ± 0.9% in mixed liquor vs 10.1% ± 0.8% in effluent) relative to the membrane-treated effluent at 5% significance level.

Comparing the percentages of ARB in mixed liquor and anoxic zone foam, no significant differences ($P > 0.05$) were observed for any of the five tested antimicrobials in MH and R2A media at SRT = 25 days. However, at the 60 day SRT, the populations of SMX^r ($P \leq 0.1$) and TMP^r ($P \leq 0.05$) bacteria exhibited elevations in foam compared to those of the mixed liquor in the R2A population (25.7% ± 3.8% SMX^r ratio in foam vs 19.9% ± 3.5% SMX^r ratio in mixed liquor, 30.8% ± 3.2% TMP^r ratio in foam vs 21.9% ± 4.0% TMP^r ratio in mixed liquor).

The overall increase in the percentage of ARB for most of the tested antibiotics from influent to mixed liquor and treated effluent of the parallel MEBPR systems (**Figures 3.6** and **3.7**) is in line with previous reports, showing that wastewater treatment contributes to an increase in the relative abundance of ARB (Ferreira da Silva et al., 2006; Lefkowitz and Duran, 2009;

Luczkiewicz et al., 2010). Increased ARB ratios in mixed liquor and effluent compared to influent can be explained by increased frequency of gene transfer facilitated at relatively long, as well as extended, SRT operations (25 and 60 day SRTs). The occurrence of genes conferring resistance to antimicrobials on mobile genetic elements (MGE) such as plasmids and integrons have been frequently documented in WWTP environments (Zhang et al., 2011; Han et al., 2012; Koczura et al., 2012; Ma et al., 2011; Mokracka et al., 2012; Rahube and Yost, 2010; Ma et al., 2013; Sentchilo et al., 2013; Szczepanowski et al., 2004b; Yang et al., 2013b; Tennstedt et al., 2003). As an example, Szczepanowski and coworkers (2009) reported the presence of more than 50 ARGs associated with resistance to the same antimicrobials tested in the present research (SMX, TET, TMP, AMX and CIP) in plasmid DNA of activated sludge and final effluent of a WWTP in Germany. Antimicrobial selective pressure, due to degradation but incomplete elimination through activated sludge treatment, could also be proposed as another cause of increased ARB ratios in MEBPR processes.

Andersson and Hughes (2010) reviewed the experimental studies associated with fitness costs of resistance and studied the rates of resistance reversibility after the antimicrobial selective pressure is reduced. Their findings suggest that in clinical settings, reversibility of resistant bacteria to susceptible bacteria is expected to be slow or non-existent. They proposed the importance of cost-free resistances, compensatory evolution and slow intrinsic resistance in reducing the driving force for displacement of ARB populations with susceptible ones.

Monitoring the role of SRT on ARB ratios, except for SMX-resistance ratio ($P \leq 0.1$) in the R2A population, no significant differences were observed in the percentages of ARB in MH and R2A mixed liquor populations at 25 and 60 day SRTs for all antimicrobials tested (**Figures 3.6** and **3.7**). A study by Walston (2013) showed that the MIC_{50} values (minimum inhibitory concentration to inhibit the growth of 50% of tested bacterial isolates) for SMX, TMP and AMP against G(+) and G(-) bacteria isolated from activated sludge of three WWTPs operating at 3, 9 and 19 days SRT were higher at the highest SRT studied. De Sotto and coworkers (2016) compared the ratio of TET^r bacteria (plated on LB agar) in SBRs at 15, 20 and 25 day SRTs. They also observed an increase in the abundance of ARB as well as EC_{50} values (concentration of TET with 50% efficiency on bacteria) in the activated sludge culturable community at longer SRT.

Previous publications have frequently reported the occurrence of AMX, SMX, TET and TMP in samples of influent, effluent and sewage activated sludge (Minh et al., 2009; Tang et al., 2009; Xu et al., 2007a; Renew and Huang, 2004; Peng et al., 2008; Li et al., 2009; Batt et al., 2007; Brown et al., 2006). In recent years, some studies have also focused on the antibiotic biodegradation potential of wastewater-isolated bacteria and the sorption behaviour of antibiotics in activated sludge treatment (Zhou et al., 2013; Herzog et al., 2013; Pérez et al., 2005; Li and Zhang, 2010). As an example, Gobel and coworkers (2005) measured the concentrations of both SMX and the fraction of SMX present as human metabolite, N^4-acetylsulfamethoxazole, in samples of raw influent, primary and secondary effluent in two WWTPs in Switzerland. Their observations indicated a reduction in the daily loads of SMX human metabolite from raw influent to primary and secondary effluent. In order to fully understand the reasons behind the selective increase/decrease in ARB percentage with long SRT operation, more comprehensive studies are required to monitor the combined correlations of antimicrobial selective pressure affected by both antimicrobial biodegradation and sorption to activated sludge and ARB ratios in MEBPR processes.

Observations from the present study also show that the MH population generally exhibited considerably higher proportions of AMX^r (42.3%-52.9% AMX^r ratio in MH bacteria vs 12.1%-32.6% AMX^r ratio in R2A bacteria), SMX^r (38.5%-50.8% SMX^r ratio in MH bacteria vs 12.5%-25.7% SMX^r ratio in R2A bacteria) and TMP^r strains (21.4%-34.6% TMP^r ratio in MH bacteria vs 9.9%-30.8% TMP^r ratio in R2A bacteria) compared to those of the R2A population (**Figures 3.6** and **3.7**). However, no significant differences ($P > 0.05$) were observed in resistance ratios of CIP between MH and R2A communities (**Figure 3.6** and **Table B.4**). It should also be noted that TET^r ratios were not statistically different beween MH and R2A tested strains of the 60 day SRT mixed liquor as well as membrane-treated effluent.

As previously described, the scope of most of the AR studies on treated effluent from WWTPs is limited to documenting the resistance profiles of single population subsets of effluent bacteria such as indicators of faecal contamination or other specific pathogenic strains (Al-Bahry et al., 2009; Okoh and Igbinosa, 2010; Ma et al., 2011; Lefkowitz and Duran, 2009; Akiyama and Savin., 2010; Zhang et al., 2009). Hence, the proportions of ARB reported in these studies are not representative of the ARB ratios in the entire population of cultivable environmental bacteria in treated effluent.

To date, various culture-based techniques have been applied to study AR in samples collected from activated sludge and treated effluent (Galvin et al., 2010; Santoro et al., 2015; Manaia et al., 2010; Reinthaler et al., 2003; Holzel et al., 2010). Although due to the high correlation between faecal indicators and human pathogens, faecal coliforms, *E. coli* and *Enterococcus* have been the most frequently studied bacterial groups in WWTPs (Mokracka et al., 2012; Rijal et al., 2009; West et al., 2011; Manaia et al., 2010; Fars et al., 2005; Akiyama and Savin, 2010; Iwane et al., 2001; Lefkowitz et al., 2010; Nagulapally et al., 2009; Reinthaler et al., 2003; Figueira et al., 2011; Mezrioui and Baleux,1994; Gallert et al., 2005; Schwartz et al., 2003; Luczkiewicz et al., 2010; Harwood et al., 2001; Martins da Costa et al., 2006), antimicrobial resistance patterns of total cultivable bacteria in the wastewater treatment processes have also been monitored in some studies (Manaia et al., 2010; Zhang et al., 2015b; Sigala and Unc, 2012; Munir et al., 2011; Gao et al., 2012; Oh et al., 2009; Huang et al., 2012). While discrepancies in the percentage of ARB among population subsets of culturable bacteria and total heterotrophs could be explained by major differences in the composition and diversity of bacterial communities, inconsistencies have also been frequently observed when similar bacterial populations (i.e. total heterotrophs) were tested for resistance to particular antimicrobials. As an example, Zhang and coworkers (2015b) documented the average ARB percentages of 40% for TET (16 µg/mL) and 81% for SMX (76 µg/mL) among R2A bacteria in the effluents of three WWTPs in Wuxi, China; however, Gao et al. (2012) reported that the relative abundances of TET^r (16 µg/mL) and SMX^r (50.4 µg/mL) R2A bacteria in the effluent samples from the East Lansing WWTP in Michigan were orders of magnitude lower. Such inconsistencies could be due to differences in conditions such as the taxonomic composition of bacterial communities in the wastewater treatment system, influent source and sludge loading rates, concentrations of antibiotics in influent and activated sludge, the concentrations of tested antimicrobials in growth assays, the choices of media (MH, R2A, LB, plate count and nutrient agar) as well as bacterial enumeration schemes such as the incubation time and temperature. In addition, the protocol for determination of the ARB ratio is also important.

Calculating the percentage of ARB could be done through two common approaches, (1) direct plating on antimicrobial-supplemented solid media, colony counting and dividing the counts of ARB by the total culturable cell counts and, (2) bacterial isolation from

antimicrobial-free media, testing for growth in the presence of antibiotics, counting the colonies with positive growth and dividing that by the total number of tested bacterial isolates. A recent publication by Neyestani et al. (2017) has reported the potential of type I error (false positive) results using direct plating techniques (first approach) in percentage of SMX^r and TMP^r bacteria due to increased concentrations of free thymidine and thymine in the wastewater environment operating at long SRT. Using the second approach (as was done in the present study) however, the following parameters could affect the representativeness of the community of tested isolates:

- the number of isolated bacteria to be tested,
- random selection of colonies. and
- the time of bacterial isolation. This could be of high importance when bacteria are isolated from media such as R2A which support the growth of both fast- and slow-growing organisms.

3.2.2 Percentage of MDR Bacteria in Influent and MEBPR Processes

To compare the MDR patterns of total cultivable heterotrophic bacteria in influent and the MEBPR environments including mixed liquor, foam and membrane effluent, the number of resistances (out of five studied antimicrobials, SMX, TMP, AMX, TET, CIP) in each of the previously tested bacterial isolates (Section 3.2.1) was first calculated. The proportion of each community with particular MDR patterns was then determined by dividing the total number of bacteria with identical number of resistances in each sample by the total number of tested viable organisms in the same sample. Results are presented in **Figures 3.8** and **3.9**. Each bar represents the mean percentage of MDR bacteria ± (standard error of the mean (SEM) of n = 3 samples collected on December 2014, January and February 2015).

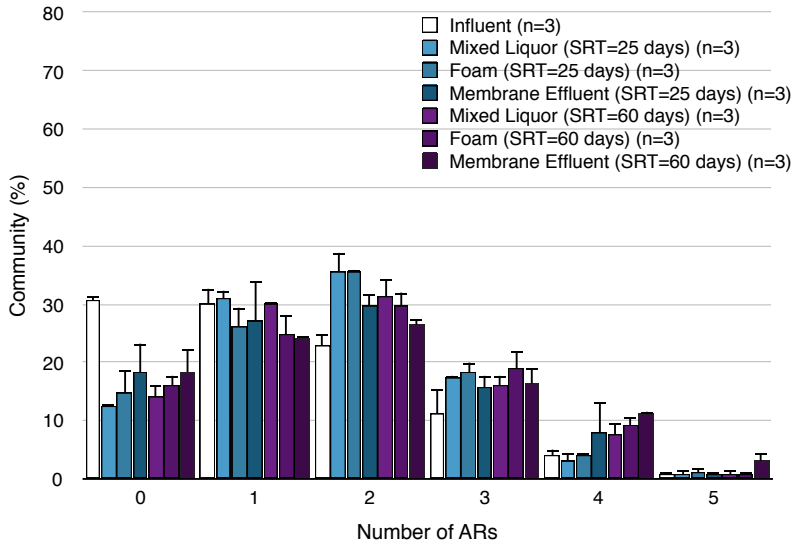


Figure 3.8 Percentage of MH bacterial community with n = 0 to 5 resistances

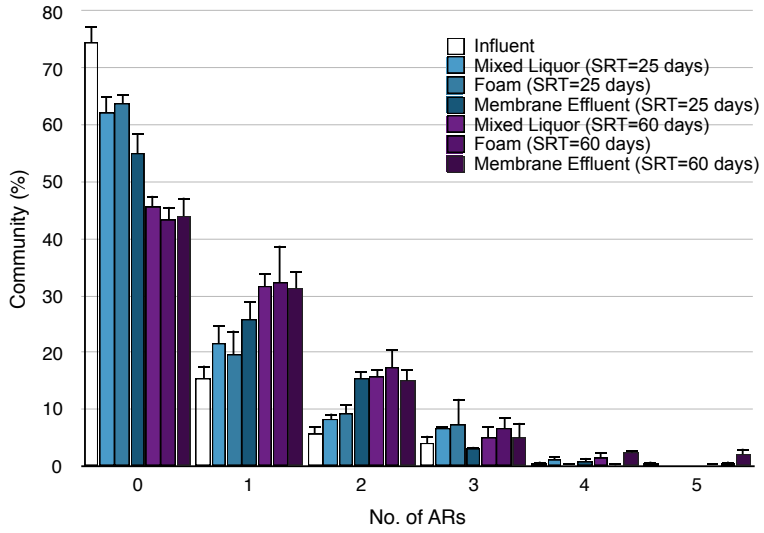


Figure 3.9 Percentage of R2A bacterial community with n = 0 to 5 resistances

As shown in **Figure 3.8**, the majority of the influent MH-tested bacteria was susceptible to all tested antimicrobials or was resistant to only one agent. However, resistances to one and two antimicrobials were the most common responses of MH bacteria in the MEBPR processes of both trains. In addition, statistical analysis showed that the MDR ratio of bacteria with four and five resistances in MH mixed liquor and membrane-treated effluent bacterial communities at the 60 day SRT train significantly increased ($P \leq 0.05$) compared to those of the mixed liquor and effluent at SRT = 25 days, respectively (**Figure 3.8**).

Similarly, **Figure 3.9** shows that parallel MEBPR processes increased the resistant (number of resistances = 1 to 5) proportions of mixed liquor, foam and treated effluent R2A groups, relative to those of the influent. Comparing the patterns of resistance between mixed liquor R2A populations, the community of resistant heterotrophs (exhibiting one and two antimicrobial resistances) was found to be significantly greater ($P \leq 0.05$) under the 60 day SRT operation (**Figure 3.9**) relative to the 25 day SRT MEBPR train. In addition to that, treated effluent R2A bacteria in the 60 day SRT system was observed to contain the highest proportion of MDR strains (9.5% ± 2.8%) compared to other compartments of the treatment process at either 25 or 60 day SRTs (**Figure 3.9**).

3.2.3 Removal of ARB by Membrane Filtration in Parallel MEBPR Trains

A summary of the average log removal of total heterotrophs as well as ARB from parallel MEBPR systems is shown in **Table 3.2**. The procedures for plating and enumeration of heterotrophic bacteria were described earlier in the Section 2.4.1.2.1. As can be seen, membrane filtration achieved three and four log reductions of total heterotrophic bacteria (growing on either MH or R2A medium) in the MEBPR systems operating at 25 and 60 day SRTs, respectively (**Table 3.2**). In addition, except for the one log decrease in removal of TET^r bacteria in the R2A community, more than 99.9% of ARB were also removed by membrane filtration from the 25 day SRT train in both MH and R2A media (**Table 3.2**). In the 60 day SRT train, however, depending on the differences in ARB ratios between influent and treated effluent samples (**Figures 3.6** and **3.7**), the log removal of ARB by membrane filtration varied between three to four logs.

Table 3.2 A a summary of the removal efficiency of total heterotrophs and ARB by membrane filtration in parallel MEBPR trains

Sample	Media	Sampling	Heterotrophic cell counts (CFU/mL) (n=9)	Average heterotrophic cell counts (CFU/mL) (n=27)	Log reduction					
					Total heterotrophs	AMX^r bacteria	CIP^r bacteria	SMX^r bacteria	TET^r bacteria	TMP^r bacteria
Membrane effluent (SRT = 25 days)	MH*	December 2014	55 (σ=11)	505 (σ=655)	3	3	3	3	3	3
		January 2015	61 (σ=19)							
		February 2015	1,402 (σ=199)							
	R2A**	December 2014	334 (σ=44)	2.2×10^{3} ($\sigma=2.7\times10^{3}$)	3	3	3	3	2	3
		January 2015	469 (σ=82)							
		February 2015	5,991 (σ=753)							
Membrane effluent (SRT = 60 days)	MH	December 2014	64 (σ=17)	63 (σ=15)	4	4	3	4	4	4
		January 2015	64 (σ=16)							
		February 2015	62 (σ=12)							
	R2A	December 2014	299 (σ=62)	317 (σ=66)	4	4	4	4	3	3
		January 2015	313 (σ=71)							
		February 2015	337 (σ=66)							

*: colony count after 16-18 hours of plating **: colony count after 36-38 hours of plating

Despite the overall high efficiency of the membrane filtration technique compared to conventional gravity settling solids-liquids separation techniques, observations from the present research show that the MEBPR train operating at 25 day SRT exhibited one log reduction of total heterotrophic bacteria compared to the 60 day SRT train (**Table 3.2**). This was due to the major membrane integrity breaches detected on February 2015 in the 25 day SRT train which resulted in substantial bacterial penetration of the permeate and that could provide the potential for microbial regrowth in the discharge piping as well. As can be seen, the average total heterotrophic counts of effluent bacteria at 25 day SRT was about seven to eight times greater than those of the 60 day SRT train.

3.2.4 Release of ARB through Waste Aerobic Mixed liquor, Foam and Membrane Effluent Discharge

To determine the release rates of resistant bacteria from MEBPR systems, daily counts of ARB (SMX^r, TMP^r, TET^r, AMX^r and CIP^r bacteria) discharged through waste aerobic mixed liquor, foam and membrane-treated effluent were calculated using **Equations 4**, **5** and **6** in Section 2.4.1.2.3. Briefly, the concentration of ARB in each environment was first determined by multiplying the average proportion of the resistant community (data presented in **Figures 3.6** and **3.7**) by the average total bacterial cell counts of the same environment (**Table B.5**). The release rate of ARB was then determined by multiplying the concentration of ARB (CFU/L or CFU/kg) by the rate of suspended solids wastage (foam (kg/day) or aerobic mixed liquor wasting rate (L/day)) or effluent flow rate (L/day)) (**Table 2.2**). It should also be noted that the outflow rate (**Equation 6**) was calculated by subtracting the total wastage of aerobic mixed liquor (L/day) from the influent daily flow rate. Release rates of ARB in waste aerobic mixed liquor, foam and effluent of parallel MEBPR systems are shown in **Figures 3.10** to **3.15**. Each bar represents the mean release rate of ARB ± (standard deviation of ARB ratio).

From **Figures 3.10** to **3.15**, SMX^r, AMX^r and TMP^r bacteria were found to be the three major types of ARB that the parallel MEBPR systems discharged through waste aerobic mixed liquor, foam and treated effluent.

In addition, the average daily release of ARB in treated effluent of the 25 day SRT train was observed to be higher (from 1.7 times for CIP to 8 times for AMX in the MH population and 3.7 times for CIP to 5.5 times for SMX in the R2A population) than the 60 day SRT permeate (**Figures 3.10** and **3.11**). It was previously discussed in Section 3.2.3 that membrane imperfections at the 25 day SRT train dramatically increased the total counts of heterotrophic bacteria in permeate samples collected on February 2015 and that affected the overall ARB removal efficiency in this train over the study period.

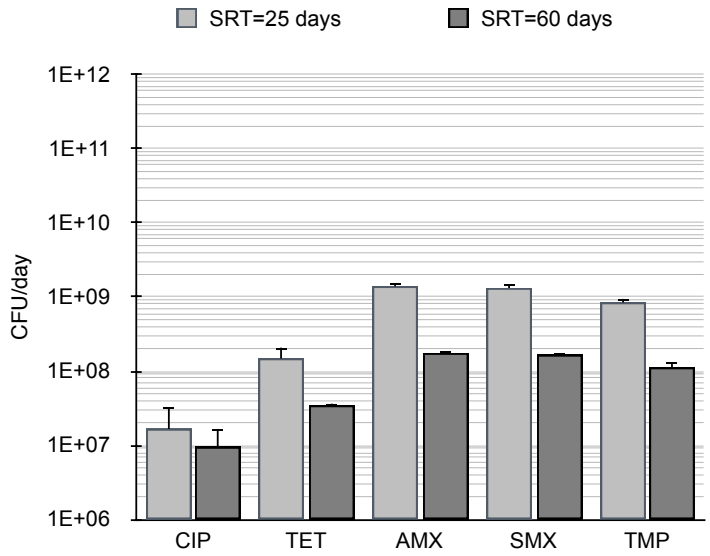


Figure 3.10 Release rates of total cultivable heterotrophic ARB in treated effluent (Medium: MH) (CFU/day)

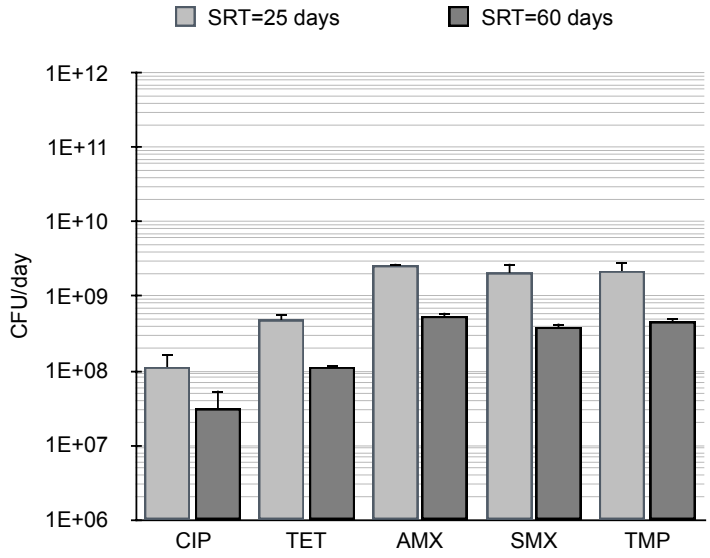


Figure 3.11 Release rates of total cultivable heterotrophic ARB in membrane-treated effluent (Medium: R2A) (CFU/day)

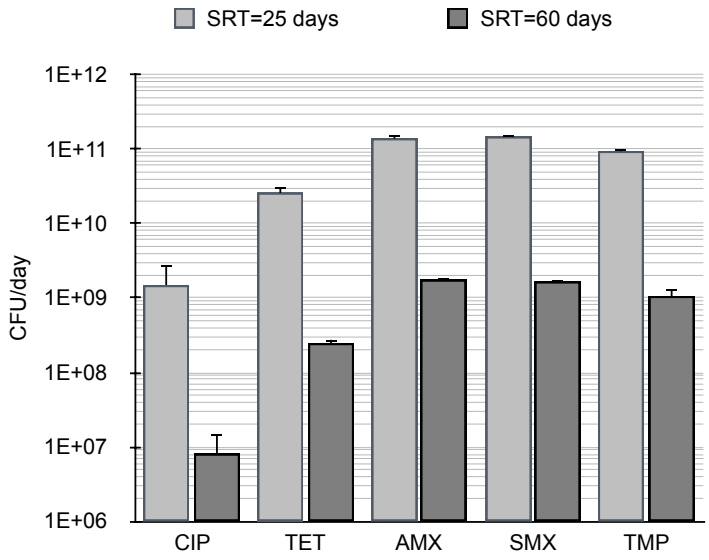


Figure 3.12 Release rates of total cultivable heterotrophic ARB in waste aerobic mixed liquor (Medium: MH) (CFU/day)

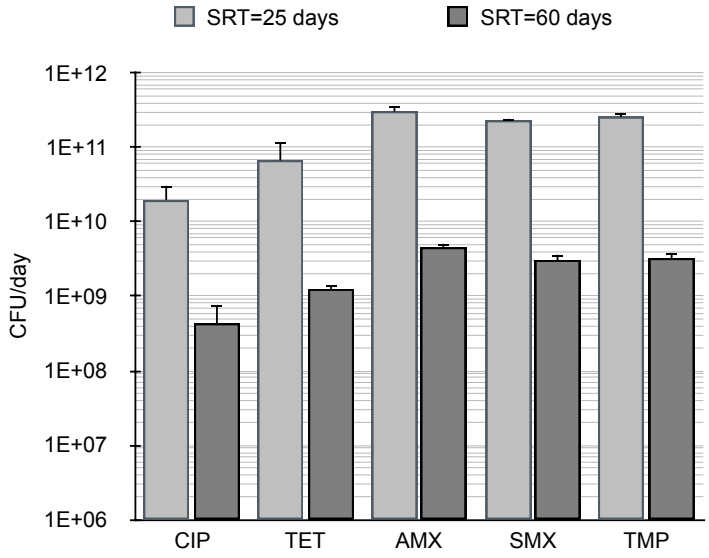


Figure 3.13 Release rates of total cultivable heterotrophic ARB in waste aerobic mixed liquor (Medium: R2A) (CFU/day)

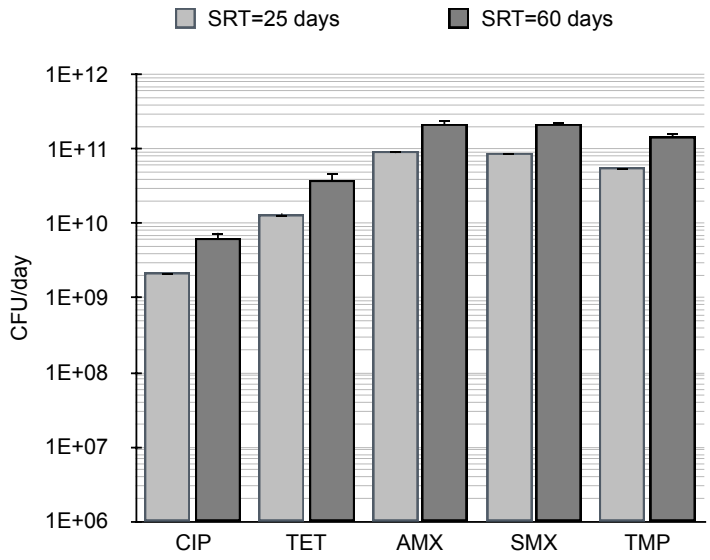


Figure 3.14 Release rates of total cultivable heterotrophic ARB in foam (Medium: MH) (CFU/day)

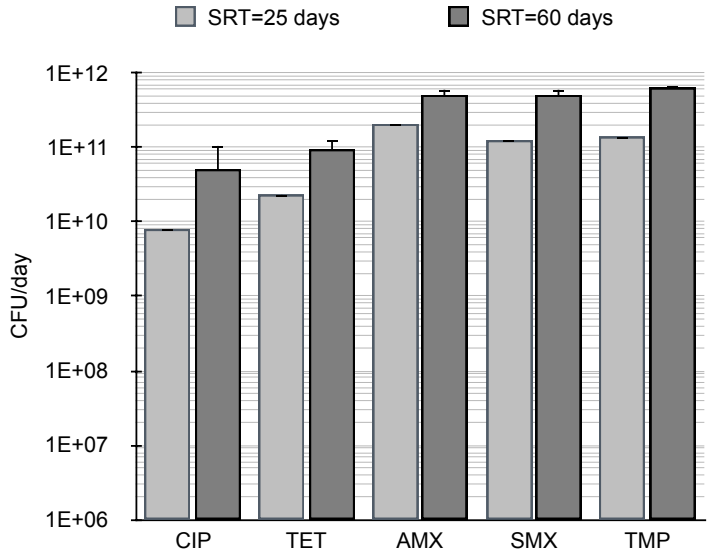


Figure 3.15 Release rates of total cultivable heterotrophic ARB in foam (Medium: R2A) (CFU/day)

From **Figures 3.12** to **3.15**, it was also observed that the release of ARB in foam at the 60

day SRT was considerably higher than that of the train operating at SRT = 25 days. Along with some significant differences in ARB proportions of R2A communities between mixed liquor and foam at the 60 day SRT train for some of the tested antimicrobials (SMX, TMP), the main reason for the larger contribution of foam in ARB discharge at the 60 day SRT train was that foam contributed only about 36% of the total daily suspended solids wastage in the MEBPR train operating at 25 day SRT; the corresponding contribution of foam at the 60 day SRT train was 99% **(Table 2.2)**. Based on similar reasoning, release rates of ARB in foam were always lower than waste aerobic mixed liquor at SRT = 25 days.

Release rates of ARB in foam and waste mixed liquor were also found to be considerably higher than those of the membrane-treated effluent at 25 and 60 day SRTs. **Figures 3.16** and **3.17** compare the ARB release ratios of waste aerobic mixed liquor and foam to membrane effluent, respectively. Release ratios of ARB were calculated by dividing the release rates of ARB in waste mixed liquor ($RR_{mixed\ liquor}$) and/ or foam (RR_{foam}) by the release rates of ARB in effluent ($RR_{effluent}$). Each bar represents the release ratios of ARB ± (standard deviation of the ARB ratio calculated using **Equation 13**).

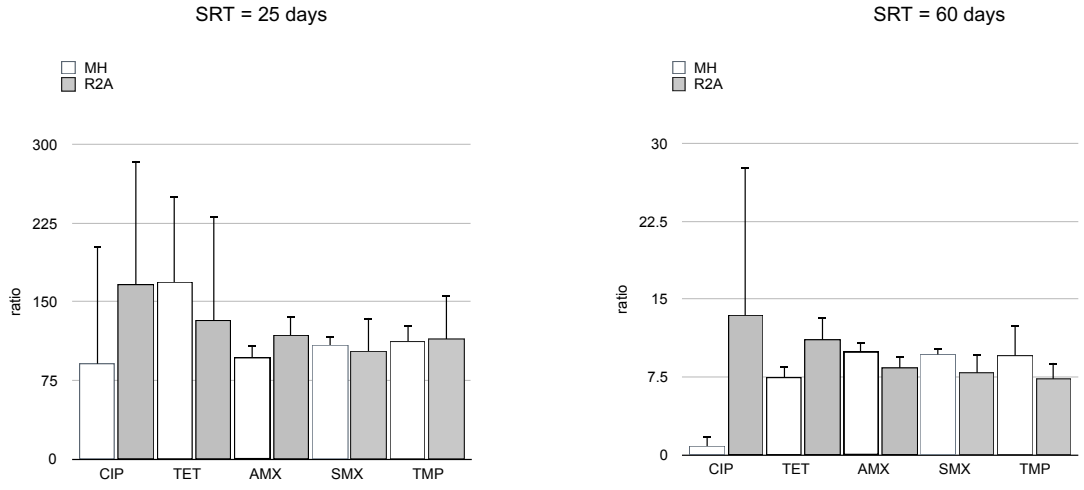


Figure 3.16 Ratios of waste aerobic mixed liquor to membrane effluent ARB release rates ($RR_{waste\ aerobic\ mixed\ liquor}/RR_{membrane\ effluent}$)

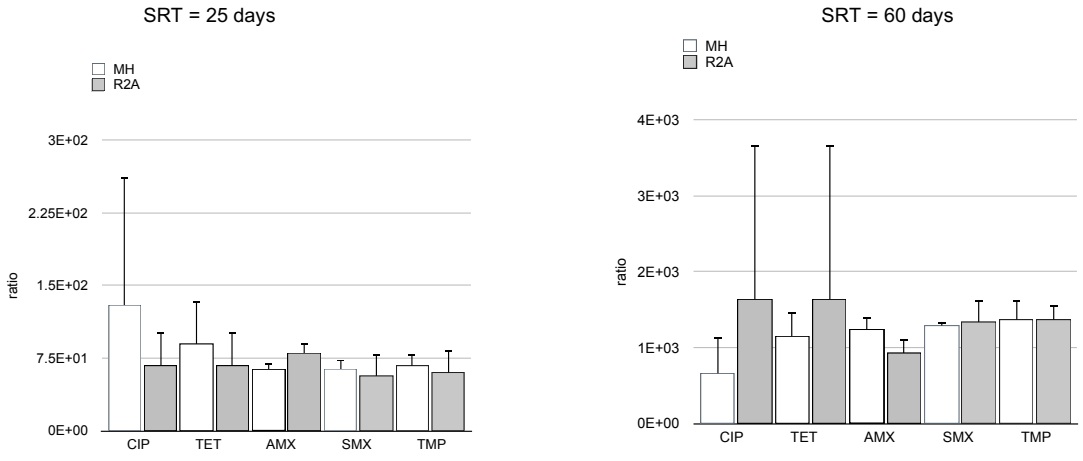


Figure 3.17 Ratios of foam to membrane effluent ARB release rates (RR_{foam} / $RR_{membrane\ effluent}$)

Observations from **Figures 3.16** and **3.17** also confirm that waste aerobic mixed liquor and foam were always the main contributors to overall ARB release from both trains of the UBC pilot plant. It should be remembered that the reductions in the ARB release ratios of waste mixed liquor to effluent ($RR_{mixed\ liquor}/RR_{effluent}$) at the 60 day SRT train was due to the reduced wastage rate of aerobic mixed liquor compared to that of the 25 day SRT. It addition to that, quantifiable differences in the ARB release ratios of foam to effluent ($RR_{foam}/RR_{effluent}$) at two different SRTs were observed which were due to the combined effects of the differences in the overall foam contribution in solids disposal as well as increased levels of ARB discharge (CFU/mL) in the permeate at the 25 day SRT train.

Table 3.3 compares the ARB release rates to the inflow rates in parallel MEBPR processes. Reported as the fraction of contribution of waste mixed liquor, foam and effluent in spread of ARB, the ratio of the ARB release rates to the ARB inflow rate was calculated using **Equations 8**, **9** and **10** in Section 2.4.1.2.3. The inflow rate of ARB (CFU/day) was determined using **Equation 3**.

Consistent with previous results (**Figures 3.10** to **3.11**), it was observed that effluents from the membrane bioreactors would make a minimum contribution ($6\times10^{-6} < F_{treated\ effluent} < 2\times10^{-3}$) to the spread of ARB to receiving environments (**Table 3.3**). Similarly, waste aerobic

mixed liquor was found to have a smaller contribution in ARB release in the train operating at SRT = 60 days, compared to that of the waste foam.

In order to study the performance of the MEBPR system operating at extended SRT (60 days) conditions with respect to ARB discharge, the ratio of total ARB release rates (RR_{Total} = $RR_{mixed\ liquor}$ + RR_{foam} + $RR_{effluent}$) to inflow rate (IR) was also determined as described in **Equation 7** in Section 2.4.1.2.3. It should be noted that the composite stanadard deviation was calculated using **Equations 13** and 14. The results are presented in **Figure 3.18**.

Table 3.3 Average ratios of ARB release rates in waste aerobic mixed liquor, foam and membrane-treated effluent to ARB inflow rates ($F_{mixed\ liquor}$, F_{foam}, $F_{treated\ effluent}$)

Media	SRT (days)	F_{AMX} (mixed liquor)	F_{CIP} (mixed liquor)	F_{SMX} (mixed liquor)	F_{TET} (mixed liquor)	F_{TMP} (mixed liquor)
MH	25	0.052	0.050	0.065	0.082	0.073
R2A		0.111	0.059	0.077	0.261	0.111
MH	60	0.0006	0.0002	0.0007	0.0008	0.0008
R2A		0.02	0.001	0.001	0.005	0.001
Media	**SRT (days)**	**$F_{AMX\ (foam)}$**	**$F_{CIP\ (foam)}$**	**$F_{SMX\ (foam)}$**	**$F_{TET\ (foam)}$**	**$F_{TMP\ (foam)}$**
MH	25	0.034	0.071	0.038	0.043	0.044
R2A		0.075	0.023	0.043	0.087	0.059
MH	60	0.082	0.207	0.095	0.126	0.119
R2A		0.182	0.152	0.176	0.37	0.269
Media	**SRT (days)**	**F_{AMX} (mixed liquor+foam)**	**F_{CIP} (mixed liquor+foam)**	**F_{SMX} (mixed liquor+foam)**	**F_{TET} (mixed liquor+foam)**	**F_{TMP} (mixed liquor+foam)**
MH	25	0.086	0.121	0.103	0.125	0.117
R2A		0.186	0.082	0.12	0.348	0.170
MH	60	0.082	0.207	0.095	0.126	0.119
R2A		0.184	0.153	0.177	0.375	0.270
Media	**SRT (days)**	**$F_{AMX\ (effluent)}$**	**$F_{CIP\ (effluent)}$**	**$F_{SMX\ (effluent)}$**	**$F_{TET\ (effluent)}$**	**$F_{TMP\ (effluent)}$**
MH	25	5×10^{-4}	5×10^{-4}	6×10^{-4}	5×10^{-4}	6×10^{-4}
R2A		9×10^{-4}	3×10^{-4}	7×10^{-4}	2×10^{-3}	1×10^{-3}
MH	60	6×10^{-5}	3×10^{-4}	7×10^{-5}	1×10^{-4}	8×10^{-5}
R2A		1×10^{-4}	9×10^{-5}	1×10^{-4}	4×10^{-4}	2×10^{-4}

From **Figure 3.18**, the ratios of the total ARB release rates to ARB inflow rates were observed to be the highest in the population of R2A bacteria in the 60 day SRT train. This is in line with previous observations on the percentage of ARB in different compartments of the treatment processes (presented in **Figures 3.6** to **3.11)** where some significant increases (SMX, TMP, AMX, TET) were observed in resistance ratios of the R2A mixed liquor population in the 60 day SRT train compared to the influent.

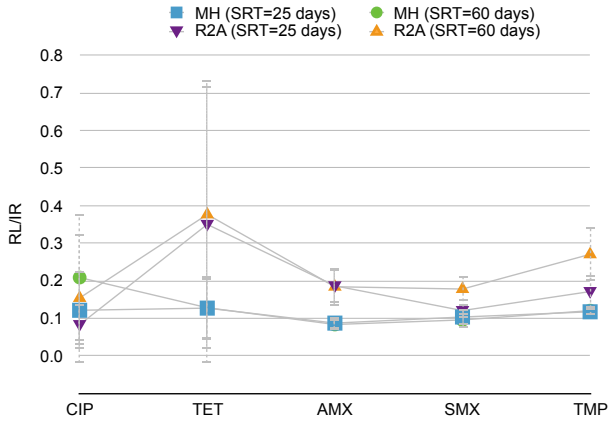


Figure 3.18 Ratios of the total release rates of ARB in MEBPR systems to ARB inflow rates (RR_{total}/ IR)

In addition, the average release rates of ARB were always lower than the ARB inflow rates meaning the number of ARB that entered the MEBPR trains through influent were greater than the daily counts of discharged ARB (**Table 3.3**, **Figure 3.18**). This implies that while MEBPR treatment processes did not reduce and sometimes even increased the ARB ratios of total heterotrophic bacteria for particular antimicrobials (**Figures 3.6** and **3.7**), the operation of these systems at 25 and 60 days of retention time was beneficial in terms of reducing the number of ARB that leave these systems. An important point to consider here is that observations in the current section are restricted to the community of cultivable heterotrophic bacteria which were able to grow in MH and/or R2A media under specific incubating conditions. Hence, the results can not represent the behavior of the true diversity of the bacterial community in these systems in the dissemination of antimicrobial resistance.

3.2.5 Characterization of Membrane-treated Effluent Cultivable Heterotrophic Bacteria in the 25 Day SRT MEBPR Train

It was noted in Sections 3.1.1 and 3.2.3 that defects in membrane filtration reduced the total cultivable heterotrophic bacterial log removal efficiency and led to the discharge of bacteria with similar and/ or increased ARB ratios compared to those that entered the MEBPR processes. In the present section, and to satisfy the research objective regarding the diversity of MH and R2A membrane-treated effluent bacteria, two methodologies were applied. First, the proportions of lactose fermenting (Lac+) bacteria in 96 MH and 96 R2A treated effluent strains from the 25 day SRT train were compared. In the second part, a total of 33 membrane effluent bacterial isolates were identified through sequencing of the 16S rRNA gene. To further characterize the community of 16S-sequenced MH and R2A heterotrophic effluent bacteria, antimicrobial susceptibility testing was performed and, the presence of *sul* resistance genes in SMX^r bacteria was assessed.

3.2.5.1 Lactose Fermentation Patterns of MH and R2A Membrane Effluent Bacteria

In order to determine the abundance of lactose fermenters (Lac+) and Gram negative (G(-)) non-lactose fermenters (Lac-) among the effluent bacteria, 96 MH and 96 R2A bacterial strains isolated from the membrane-treated effluent of the 25 day SRT train (previously tested for resistance to SMX, TMP, TET, AMX and CIP in Section 3.2.1) were selected and their growth characteristics were analyzed on MacConkey agar.

A detailed description of the materials and methods used for lactose fermentation testing assays in the present project was provided in Section 2.4.1.3. It should be noted that to ensure broad representation of MH and R2A communities for the purpose of lactose fermentation testing, 192 effluent bacteria were randomly selected considering that the ARB percentages in each group (MH and R2A) were not considerably different from those of the total-tested effluent bacteria presented in **Figures 3.6** and **3.7**.

The percentages of Lac+ and G(-) Lac- bacteria among the MH and R2A membrane-treated effluent bacteria are presented in **Figure 3.19**. The relative abundance of each community

was determined by dividing the total number of members in each group (Lac+ or Lac-) by the total number of tested strains (n=96). In addition, each of the two groups of Lac+ and Lac- effluent bacteria were classified into five sub-groups based on the number of resistances (n = 0 to 4).

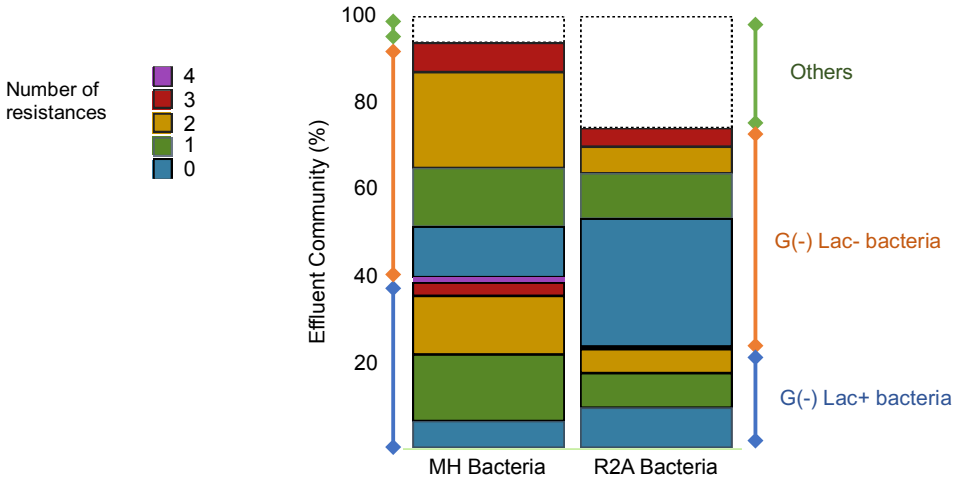


Figure 3.19 Comparison of the lactose fermenting communities of MH and R2A effluent tested bacterial isolates (n=192)

As can be observed in **Figure 3.19**, the percentage of Lac+ bacteria in the MH community was 39.5%, the corresponding value in R2A population was 23.9%. In addition, the percentage of G(-) Lac- bacteria was similar in both media (54.16% in the MH population vs 50.0% in the R2A group). As crystal violet and bile salts are inhibitors to most species of G(+) bacteria, it was expected that the proportion of bacteria which did not grow on MacConkey medium was mostly representative of the community of G(+) bacteria. It has also been shown that some fastidious G(-) strains (e.g. *Pasteurella multocida*) cannot grow on this medium (Hamilton and Larsen, 2008). As can be seen in **Figure 3.19**, 6.25% of the MH population and 26.04% of the R2A group belonged to this group named as “others”.

Figure 3.19 shows that in the MH effluent population, a greater percentage of dual-resistant bacteria (21.88% in Lac- bacteria vs 13.54% in Lac+ bacteria) and MDR bacteria (7.29% in Lac- bacteria vs 3.13% in Lac+ bacteria) belonged to the group of G(-) Lac- bacteria relative

to those of the Lac+ population. Similarly, the R2A G(-) Lac- bacterial group included a larger population of bacteria with three resistances (4.17% vs 1.04%) compared to that of the Lac+ group. In addition, and consistent with the observations from **Figures 3.8** and **3.9**, the MH population exhibited greater percentages of dual-resistant and MDR bacteria compared to that of the R2A community.

Despite the low number of tested strains in the lactose fermentation testing assay (n=192), observations from **Figure 3.19** suggest that the differences observed in the percentage of ARB (SMX, TMP, TET, AMX, CIP) as well as MDR patterns in Section 3.2.1 between MH and R2A communities (**Figures 3.6** to **3.9)** could be caused by monitoring the ARB growth in two different bacterial compositions which were isolated on each medium.

To study what types of ARB exhibited the highest abundance within each group, the total number of Lac+ (or Lac-) bacteria with positive growth in antimicrobial-supplemented media was divided by the total number of Lac+ (or Lac-) bacteria in antimicrobial-free media. Results are presented as the percentage of ARB in **Figure 3.20**. Comparing the resistance ratios of ARB between the two subgroups of the MH population, the G(-) Lac- group exhibited a higher proportion of AMX^r and TMP^r bacteria (61% vs 50% in AMX and 32% vs 26% in TMP) and a lower proportion of SMX^r bacteria (42% vs 55%) compared to the lac+ bacteria. In the R2A population, however, a larger proportion of Lac+ bacteria was resistant to AMX (39% vs 18%) compared to G(-) Lac- bacteria.

It addition, both the R2A Lac+ and G(-) Lac- bacterial groups were observed to have lower percentages of AMX^r, SMX^r and TMP^r bacteria compared to those of the MH population (**Figure 3.20**). This could be due to the formulation of R2A medium in which the low levels of nutrients permit the growth of a larger community of oligotrophic environmental bacteria from activated sludge which were sensitive or resistant to fewer antimicrobials compared to the MH population.

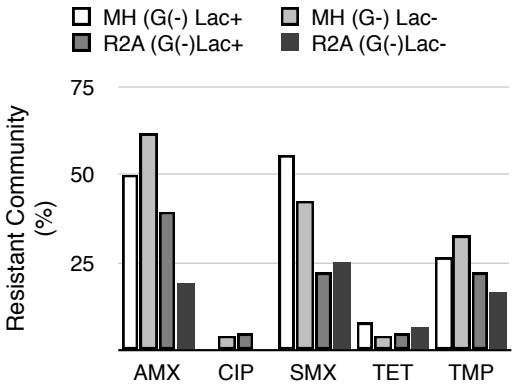


Figure 3.20 Percentage of ARB within subgroups of Lac+ and G(-) Lac- membrane effluent bacterial isolates (n=192)

In order to determine the contribution of Lac+ and G(-)Lac- effluent bacteria to the total ratios of ARB, the total number of Lac+ (or Lac-) bacteria with positive growth in antimicrobial-supplemented media was divided by the total number of tested bacteria (n = 96) in antimicrobial-free media. Data are presented in **Figure 3.21**.

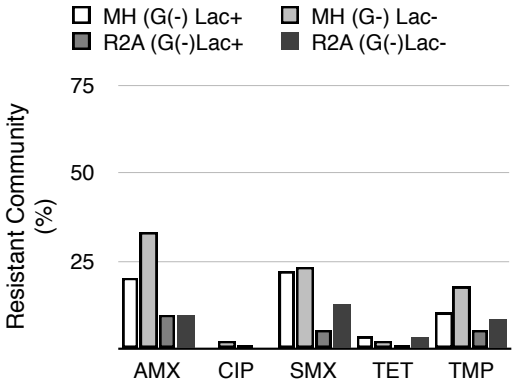


Figure 3.21 Percentage of ARB in membrane-treated effluent bacterial isolates (n = 192)

As can be seen, compared to Lac+ bacteria, MH G(-) Lac- bacteria made a greater contribution to AMX and TMP resistance (33% vs 20% in AMX and 11% vs 18% in TMP).

However, MH Lac+ and G(-) Lac- bacteria were present in similar proportions to SMX resistance in treated effluent bacteria. It was also observed that R2A G(-) Lac- bacteria made a larger contribution to SMX resistance (12.5% vs 5%) compared to Lac+ bacteria (**Figure 3.21**).

In general, comparison of the communities of MH and R2A membrane-treated effluent bacterial isolates by monitoring growth in liquid cultures as well as lactose-based assays showed that MH medium not only favored the growth of a larger population of lactose fermenters (Lac+), but also exhibited a greater proportion of MDR bacteria in both Lac+ and G(-) Lac- groups compared to the R2A medium. Given that enteric bacilli commonly grow and replicate in the intestinal tract of humans and are destroyed outside their host to varying degrees, it is unexpected to find a large population of them in mixed liquor and treated effluent in WWTPs operating at long SRTs. Hence, despite knowing that the culture-based techniques are not capable of providing a true representative diversity of microbial communities due to limitations of this technique in bacterial enrichment and growth in cultivation medium, R2A could be suggested as a more suitable medium for isolation of a larger proportion of G(-) Lac(-) environmental bacteria from membrane-treated effluent. In addition, as the highest abundance of dual-resistant and MDR effluent bacteria were found in the group of G(-) non-lactose fermenters in both MH and R2A communities (**Figure 3.19**), it is recommended that evaluating the efficiency of treatment plant solids-liquid separation techniques in ARB removal is not limited to determination of the log removal of fecal contamination indicators such as *E. coli* or total coliforms.

3.2.5.2 Identification of Membrane-treated Effluent Bacterial Isolates by 16S rRNA Gene Sequencing

Bacterial growth on selective and differential MacConkey agar (lactose-based method) provided an opportunity for the quick determination and comparison of the relative abundances of lactose fermenting (Lac+) bacteria and G(-) non-lactose fermenting (Lac-) bacteria in MH and R2A membrane effluent communities (Section 3.2.5.1). However, compared to classical methods of bacterial identification based on morphological traits and biochemical properties (such as lactose fermentation), the use of genetic techniques, such

as sequence analysis of the 16S rRNA gene, facilitates rapid and accurate identification of microorganisms (Cook et al., 2003).

In the present study, 16S rRNA gene sequencing was performed to introduce a list of Lac- and Lac+ strains that had been isolated from the membrane-treated effluent in the MEBPR train at SRT = 25 days. It should be noted that due to time and cost constraints, 16S rRNA gene sequencing of the total number of tested-bacteria from the lactose-based method (96 MH and 96 R2A tested strains) was not possible and hence, only 16 MH and 17 R2A membrane-treated effluent bacterial isolates were identified at the genus level. It should be noted that the selection of 33 bacterial isolates was done to cover (1) the most different-looking colonies (including pigementation, shape and size) and (2) the most diverse AR phenotypes against five tested antimicrobials (SMX, AMX, TET, CIP and TMP).

A descriptive summary of the methodology for preparation of crude bacterial cell lysates and PCR amplification, as well as 16S-sequencing analysis, was provided in Sections 2.4.2.1 and 2.4.2.2. **Figures 3.22** and **3.23** represent the phylogenetic trees corresponding to the identified MH and R2A bacterial isolates built with the sequences of the V3 region of the 16S rRNA gene.

The results indicate that G(-) non-lactose fermenters, such as bacteria affiliated with the genera *Acinetobacter*, *Aeromonas*, *Pseudomonas*, and *Stenotrophomonas* were commonly detected in MH and R2A bacterial communities (**Figures 3.22** and **3.23**). However, while Lac- *Shigella*, *Pseudoxanthomonas* and *Brevundimonas* were only identified in the MH group, bacteria affiliated with the genera *Chryseobacterium*, *Comamonas*, *Delftia*, and *Deinococcus* were only isolated from the R2A-tested community. Similarly, in both MH and R2A effluent populations, bacteria affiliated with the genera *Bacillus* and *Enterococcus* were identified, which are well-characterized members of the G(+) bacteria.

In the present project, a total of 41 MH and 31 R2A heterotrophic bacteria (influent and membrane effluent) were identified through 16S rRNA gene sequencing analysis and a summary of these, as well as their corresponding isolating medium, are presented in **Table 3.4**.

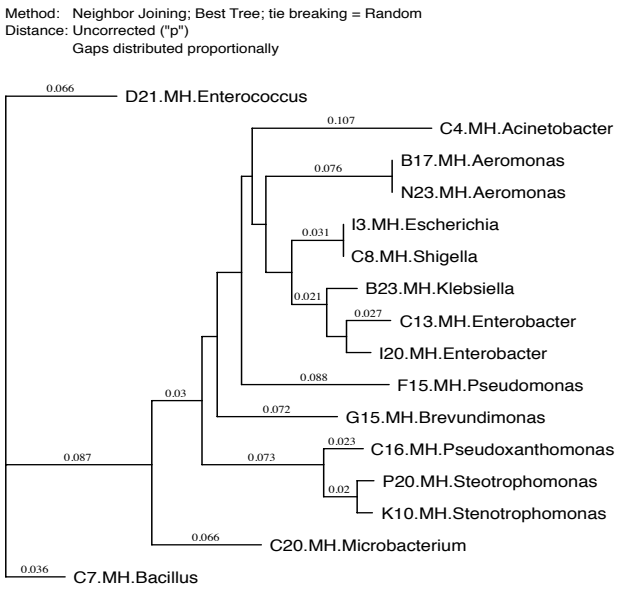


Figure 3.22 Phylogenetic tree of MH membrane-treated effluent bacterial isolates

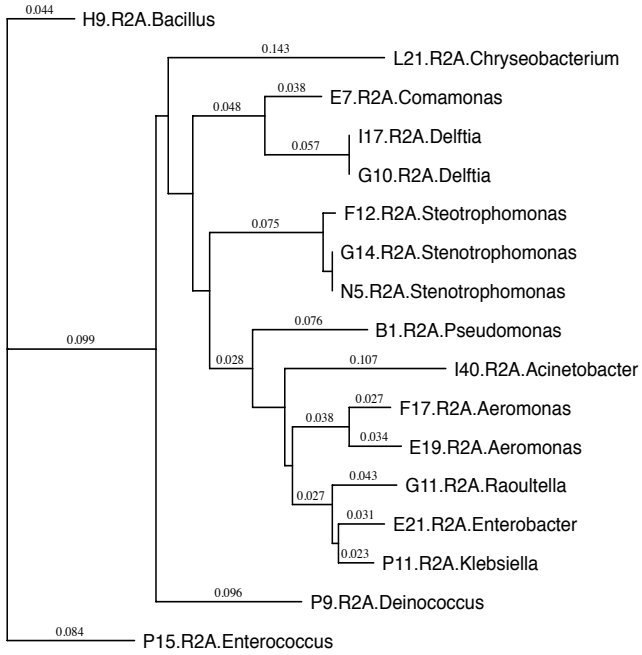


Figure 3.23 Phylogenetic tree of R2A membrane-treated effluent bacterial isolates

Table 3.4 A summary of the 16S-sequenced influent and treated effluent bacterial isolates and corresponding isolating media

Phylum	Class	Order	Family (Strain)	Isolating Medium
Proteobacteria	*Alpha-proteobacteria*	*Caulobacterales*	*Caulobacteraceae* *(ER2A-20, MH-G15)*	MH (n=1) R2A (n=1)
		Sphingomoadales	*Sphingomonadaceae* *(ER2A-29)*	R2A (n=1)
	Beta-protobacteria	*Burkholderiales*	*Comamonadaceae* *(EMH-22, EMH-21, R2A-I17, R2A-G10, R2A-E7, IMH-20)*	MH (n=3) R2A (n=3)
			Burkholderiaceae *(IMH-29)*	MH (n=1)
		Neisseriales	*Neisseriaceae* *(IMH-24)*	MH (n=1)
	Gamma-proteobacteria	*Enterobacteriales*	*Enterobacteriacea* *(MH-I3, MH-B23, MH-I20, MH-C13, MH-C8, R2A-E21, R2A-P11, R2A-G11, EMH-17, ER2A-23, ER2A-3, EMH-11, EMH-25, IMH-11, IMH-26, IMH-3, IMH-14, IMH-30, IMH-6, IR2A-3, IMH-19, IR2A-6)*	MH (n=15) R2A (n=7)
		Aeromonadales	*Aeromonadaeae* *(EMH-6, EMH-10, ER2A-10, MH-B17, MH-N23, R2A-F17, R2A-E19, IMH-10)*	MH (n=5) R2A (n=3)
		Xanthomonadales	*Xanthomonadaceae* *(MH-C16, MH-P20, MH-K10, R2A-F12, R2A-N5, R2A-G14, EMH-5, EMH-14, EMH-29, IR2A-11)*	MH (n=6) R2A (n=4)
		Alteromonadales	*Shewanellaceae* *(EMH-2, EMH-9)*	MH (n=2)
		Pseudomonadles	*Moraxellaceae* *(MH-C4, R2A-I40, IR2A-25)*	MH (n=1) R2A (n=2)
			Pseudomonadaceae *(ER2A-17, ER2A-30, EMH-20, EMH-30, R2A-B1, MH-F15)*	MH (n=3) R2A (n=3)
Bacteriodetes	*Flavobacteria*	*Flavobacteriales*	*Flavobacteriaceae* *(ER2A-15, R2A-B1, IR2A-20)*	R2A (n=3)
Firmicutes	*Bacilli*	*Lactobacillales*	*Enterococccea* *(MH-D21, R2A-P15)*	MH (n=1) R2A (n=1)
		Bacillales	*Staphylococcaceae* *(ER2A-24)*	R2A (n=1)
			Bacilliaceae *(MH-C7, R2A-H9)*	MH (n=1) R2A (n=1)
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Microbacteriaceae* *(MH-C20)*	MH (n=1)
Deinococcus-Thermos	*Deinococci*	*Deinococcales*	*Deinococcaceae* *(R2A-P9)*	R2A (n=1)

It can be seen in **Table 3.4** that bacteria affiliated with the orders *Caulobacteriales,*

Burkholderiales, Enterobacteriales, Aermonadales, Xanthomonadales, Pseudomonadales, Lactobacillales and *Bacillales* could comonly grow on either MH or R2A medium. However, while bacteria affiliated with the family *Niesseriaceae*, *Shewanellaceae* and *Microbacteriacea* were only isolated on MH medium, strains from four families of *Flavobacteriaceae, Deinococcaceae, Sphingomonadaceae* and *Staphylococcaceae* were only isolated on R2A medium. Although the low number of 16S-sequenced bacterial isolates (n= 72) in the present project is not adequate for comprehensive comparison of the diversity of bacterial isolates on each medium, our data suggest that the low-nutrient R2A medium could be used to isolate diverse phyla of environmental bacteria, including *Bacteroidetes* and *Deinococcus-Thermos.*

Gensberger and coworkers (2015) evaluated the effects of medium type and temperature on the composition of water microbial communities collected from water wells in Lower Australia by 16S rRNA gene sequence analysis of clone libraries. They observed that cultivation on R2A yielded more biodiverse populations of heterotrophic bacteria compared to high-nutrient yeast extract medium. In order to fully investigate the taxonomic composition of MH and R2A bacteria, 16S rRNA gene sequencing should be done on representative bacterial populations of WWTPs isolated from each medium and phylogenetic trees should be compared.

3.2.5.2.1 MDR Patterns of the 16S-sequenced Membrane Effluent Bacterial Isolates and Detection of the *sul* Genes in SMX^r Effluent Bacteria

Due to the importance of the release of MDR bacteria in WWTP effluent, antimicrobial susceptibility patterns of 33 16S-sequenced membrane effluent strains against a set of 16 new antimicrobials (different from the five previously-tested agents) were also determined (**Table 3.5)**. As observed, the community of MDR bacteria increased from 33.3% (while testing five antimicrobials in Section 3.2.1) to 75.7%, when susceptibilities to 21 antibiotics were monitored (**Table 3.5**). This implies that the percentage of MDR bacteria is a function of the number of tested antimicrobials and hence, the abundance of MDR bacteria in environmental samples should not be underestimated due to limitations in the number of tested antimicrobial agents.

Table 3.5 Antimicrobial susceptibility profiles of 16S-sequenced membrane effluent bacterial isolates (presented in Section 3.2.5.2)[a]

Genus	Strain	AR Phenotype	Aminoglycosides				Quinolones		β-Lactams				Tetracyclines		COL 10	TMP 5	SXT 25	CM 30
			STR10	GEN10	AMK30	KAN30	CIP5	NAL30	AMC30	AMP10	CFM5	IMP10	TET30	DOX30				
Stenotrophomonas	MH-P20*	AMX,TET TMP	•	•					•	•	•		•			•		
	MH-K10*	AMX,TMP	•						•	•	•	•				•		
	R2A-F12*	AMX,TET, TMP	•			•			•	•	•	•	•			•		
	R2A-N5*	TET,SMX, AMX							•	•	•	•	•		•			
	R2A-G14*	AMX,TMP	•	•					•	•	•	•				•		
Aeromonas	MH-B17*	AMX,SMX TET							•	•			•					
	MH-N23	AMX,SMX							•	•								
	R2A-F17*	AMX,SMX TET							•	•			•					
	R2A-E19	AMX,SMX							•	•								
Bacillus	R2A-H9*	AMX,TMP							•	•	•				•	•		
	MH-C7*	AMX,TMP SMX								•	•				•	•	•	
Enterobacter	MH-C13	AMX,SMX							•	•								
	MH-I20*	AMX,TET, TMP							•	•						•		•
	R2A-E21*	AMX,TMP, TET							•	•						•		•
Delftia	R2A-I17*	AMX,AMK TMP	•	•	•	•			•	•	•	•			•	•		
	R2A-G10*	AMX	•	•	•	•				•					•			
Enterococcus	R2A-P15*	-	•					•			•				•			
	MH-D21*	TET	•	•	•	•					•		•		•			
Pseudomonas	R2A-B1	TMP								•	•					•		
	MH-F15*	TMP,SMX								•	•					•	•	
Klebsiella	MH-B23*	SMX,TET, TMP								•			•	•		•	•	•
	R2A-P11*	CIP,TMP					•	•		•	•				•	•		
Pseudoxanthomonas	MH-C16*	AMX, TMP	•	•	•	•				•	•					•		
Ecoli	MH-I3*	AMX,SMX TET,TMP							•	•			•			•	•	
Microbacterium	MH-C20*	TMP	•	•	•	•										•		

Genus	Strain	AR Phenotype	Aminoglycosides				Quinolones		β-Lactams				Tetracyclines		COL 10	TMP 5	SXT 25	CM 30
			STR10	GEN10	AMK30	KAN30	CIP5	NAL30	AMC30	AMP10	CFM5	IMP10	TET30	DOX30				
Brevundimonas	MH-G15*	CIP,TMP	•				•	•		•					•	•		
Acinetobacter	MH-C4	TMP														•		
Shigella	MH-C8	CIP					•	•										
Comamonas	R2A-E7*	SMX,TMP	•			•					•				•	•	•	
Acinetobacter	R2A-I40*	TMP,SMX	•	•											•	•	•	•
Deinococcus	R2A-P9	TMP,SMX														•	•	
Raoultella	R2A-G11	SMX								•								
Chryseobacterium	R2A-L21*	SMX								•	•				•			

[a] : Filled black circles indicate resistance (diameter of the zone of inhibition ≤ one centimeter); the absence of a circle mark indicates a larger inhibition zone. * :MDR bacteria

AMK: Amikacin, GEN: Gentamicin, KAN: Kanamycin, STR: Streptomycin, CIP: Ciprofloxacin, NAL: Nalidixic acid, DOX: Doxycycline, TET: Tetracycline, AMC: Amoxicillin/clavulanic acid, AMP: Amplicillin, IMP: Imipenem, CFM: Cefixime, COL: Colistin, TMP: Trimethoprim, CM: Chloramphenicol, SXT: SMX+TMP

Comparing the susceptibility results of membrane effluent bacteria to antibiotics in different classes (**Table 3.5**), co-resistance was also observed in some cases. As an example, 13 out of 15 SMX^r strains, 17 out of 21 TMP^r strains and all TET^r strains (n=10) were found to be resistant to at least one of the antibiotics in the class of β-Lactams. A potential reason for antimicrobial co-resistance may be the presence of a particular resistance mechanism (e.g. multidrug efflux pump) which could be responsible for resistance to different classes of antibiotics (Chopra and Roberts, 2001).

Characterization and identification of total heterotrophic effluent bacterial isolates in the present research provided information on the diversity and the potential release of MDR cultivable heterotrophs from the MEBPR process at 25 day SRT. However, it is not known whether the identified community of cultivable bacteria represents a small or a large fraction of the total bacterial community of membrane-treated effluent. Using Illumina Miseq sequencing data, the proportion of the identified isolated taxa (Section 3.1.4 and 3.2.5.2)

relative to the total community (culturable and uncluturable microbial communities) of treated effluent bacteria was determined as presented in section 3.3.1.

The presence of two sulfonamide resistance genes (*sul*1 and *sul*2) in total genomic DNA of influent and membrane effluent of the 25 day SRT MEBPR train was previously reported in Section 3.1.5. The final aspect that was studied in the community of cultivable heterotrophic effluent bacteria was the identification of ARGs responsible for observed resistance to SMX and the determination of the proportion of effluent bacterial community carrying a particular gene. Briefly, the occurrence of two sulfonamide genes (*sul*1 and *sul*2) was tested in 16S-sequenced SMX^r effluent bacteria by PCR-based examination (**Table B.6**). A summary of the DNA extraction procedure and a list of primers and PCR conditions to amplify the genes of interest was provided in Sections 2.4.2.1 and 2.4.2.2. In total, out of 15 tested bacteria, the *sul*1 gene was detected in nine strains (60%) and the *sul2* gene was detected in eight bacterial isolates (53%). In addition, the co-occurrence of *sul*1 and *sul2* genes was detected in two bacterial isolates. The similarity between the relative abundances of the *sul*1 and *sul*2 genes (60% vs 53%) in culturable effluent bacterial isolates highlights the possibility that the *sul*2 gene could also be present at similar relative abundances (gene copies normalized to the 16S rRNA gene copies) in MEBPR processes. Understanding the fate of ARGs in wastewater treatment systems requires a comprehensive monitoring of all known genes conferring resistance to an antimicrobial of interest. However, antibiotics such as β-lactams, trimethoprim (TMP) and tetracyclines have multiple cassate-associated ARGs; measuring the relative distribution of all in a single study is not manageable due to time and cost limitations. Hence, the scope of studies is usually limited to measuring the concentrations of a limited number of ARGs in WWTPs (Börjesson et al., 2009; Gao et al., 2012; Lachmayr et al., 2009; Grape et al., 2007).

In general, 16S rRNA gene sequencing analysis of 33 effluent bacterial isolates from the MEBPR train at the 25 day SRT identified a total of 12 families of bacteria, the majority of which (75.7%) exhibited MDR as confirmed by antimicrobial susceptibility testing. In other research, the impacts of WWTP effluent discharges in natural receiving waters have been extensively studied and increased populations of ARB and ARGs downstream of the WWTPs discharge points have been frequently reported (Goñi-Urriza et al., 2000; Iwane et al., 2001; Li et al., 2010; LaPara et al., 2011; Yang et al., 2013a). Observations from this

section of the present study emphasize the need for regular monitoring of the quality of membrane effluent in terms of the occurrence and abundance of ARB and ARGs and to minimize the impacts of unexpected release of resistant bacteria and associated genes, particularly in cases where membrane filtration is used as the final stage prior to effluent discharge in the environment.

In the present research, ARB patterns of total cultivable heterotrophic bacteria in influent and parallel MEBPR trains of the UBC pilot plant were compared. A summary of conclusions in Section 3.2 includes the following.

- The results indicated that the parallel MEBPR systems significantly increased the percentages of ARB (SMX^r, TMP^r) as well as MDR bacteria relative to the influent as observed from the comparison of ARB proportions between influent and the processes redox zones. Statistically significant increases were also observed in the populations of SMX^r ($P \leq 0.1$), TMP^r ($P \leq 0.05$) and TET^r ($P \leq 0.1$) R2A bacteria in foam compared to mixed liquor in the 60 day SRT train.
- In addition, with the exception of SMX^r ratio in the R2A population, the proportions of ARB (TMP, AMX, TET, CIP) in the mixed liquors of 25 and 60 day SRT trains were not significantly different implying that extending SRT from 25 to 60 days did not adversely impact the antimicrobial resistance patterns of heterotrophic bacteria inside the MEBPR systems.
- Observations from the present study highlighted the point that operation of the MEBPR systems at 25 and 60 day SRTs reduced the average ARB discharge daily counts relative to the influent ARB daily counts. This was determined through comparison of the total release rates of cultivable heterotrophic resistant bacteria through waste aerobic mixed liquor, foam and effluent discharge with ARB inflow rates.
- The current research also showed that foam from the 60 SRT MEBPR system (average 90.1% to 99.8% contribution) and the waste aerobic mixed liquor at the 25 day SRT train (average 41.2% to 74.6% contribution) were the major contributors to the release of ARB in parallel MEBPR systems.

- With the exception of TET^r bacteria in the R2A group, membrane filtration in parallel MEBPR processes always achieved more than three log reductions of ARB. In addition, considerable increases in ARB and total heterotrophic counts of effluent bacteria were observed due to membrane integrity issues at the 25 day SRT train over the period of study, relative to the 60 day SRT system.
- 16S rRNA gene sequence analysis helped identify a set of 33 antibiotic resistant bacteria from 12 families of bacteria in the 25 day SRT train permeate. Differences in the relative abundance of Lac+ bacteria between the MH and R2A effluent cultivable heterotrophic bacterial communities were also observed using lactose-based methods.
- It should be remembered that, in evaluation of the role of wastewater treatment processes in dissemination of AR using culture-dependent approaches, a large number of factors are involved which set limitations on the ability to evaluate the treatment processes performance. A summary of the most important variables which complicate our assessment of the role of WWTPs in ARB release is summarized in **Figure 3.24**. As can be observed, apart from the importance of (1) system operating conditions (e.g. solids retention time (SRT), hydraulic retention time (HRT)) which directly affect the bacterial growth/decay rates, (2) system configurations which influence the taxonomic composition of bacterial communities in the wastewater treatment process and (3) the ARB discharge pathway chosen for study (e.g. effluent, mixed liquor, foam), (4) the cultivable bacterial community of interest (e.g. total heterotrophs, faecal coliforms) as well as (5) the antimicrobial resistances (including the antibiotics and antibiotic resistance genes (ARGs)) that are specified to be of research interest, are of critical importance.

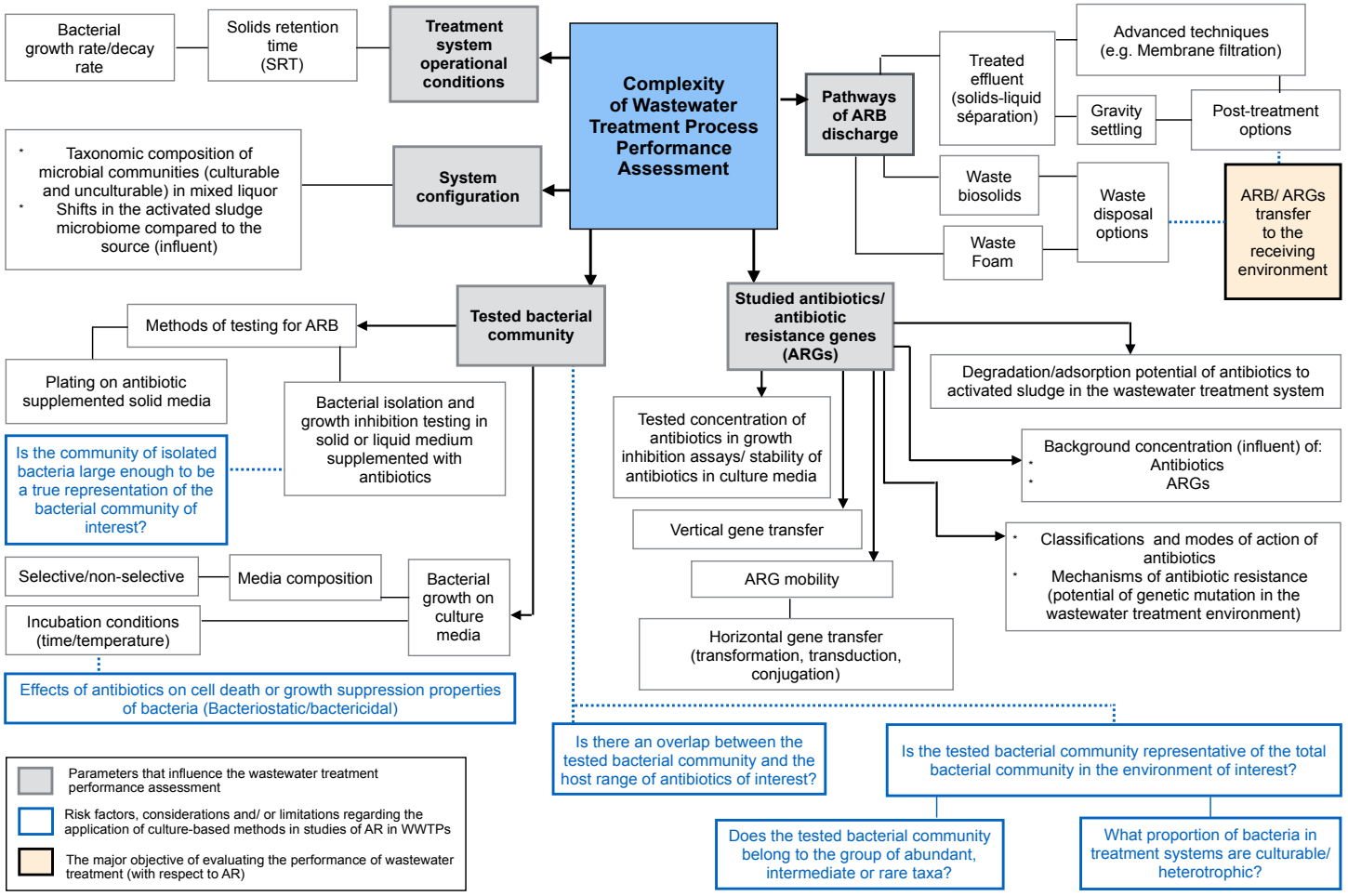


Figure 3.24 Complexity of the wastewater treatment process performance assessment using culture-dependent techniques

As an example, one of the most important considerations regarding the application of culture-based methods in studies of AR in total heterotrophic bacteria in WWTPs is to know the limitation of this technique in isolating a representative community of bacteria in the environment of interest. The most critical factors which influence the diversity of tested bacterial community on culture media include (1) media composition (2) incubation conditions (including time and temperature) and (3) the methodology for deterimation of ARB ratio (including direct bacterial plating and/or bacterial isolation which could influence the representativeness of the bacterial community of interest).

Observations from Sections 3.2.1 to 3.2.4 highlight the point that evaluation of the performance of WWTPs through comparison of the ARB release rates to ARB inflow rates is strongly dependent on (1) the tested antimicrobials (as observed by differences in ARB ratios), (2) the choice of growth medium (MH vs R2A) and (3) SRT as a major system operating parameter of the wastewater treatment process. Hence, our assessment of the role of long SRT operations in attenuation of AR in MEBPR systems should always be limited to the studied antimicrobial resistance, as well as the community of tested organisms.

3.3 Assessment of Bacterial Communities in a Model MEBPR System and Identification of ARGs in the Foam Microbiome

The adoption of membrane bioreactor (MBR) technology overcomes biomass settleability problems and provides a potential to maintain high concentrations of suspended solids in mixed liquor and to operate wastewater treatment systems at long solids retention times (SRTs). However, studies have shown that coupling of MBR technology with enhanced biological phosphorus removal (EBPR) processes could proliferate foaming on the surface of the anoxic zone mixed liquor and impose adverse effects on the operational integrity of the process (Monti, 2006). Hence, regular harvesting of foam has been proposed as an efficient strategy not only to reduce the accumulation of foam, but also to control SRT in the UBC pilot plant MEBPR systems (Hall et al., 2011; Yue, 2017).

In Section 3.2, the release of antibiotic resistant cultivable heterotrophic bacteria in waste

aerobic mixed liquor, foam and membrane-treated effluent in MEBPR systems operating at 25 and 60 day SRTs was assessed. It was shown that at very long SRT (60 days), foam was the major contributor (90.1%-99.8% contribution) to the release of antibiotic resistant bacteria (ARB) from the MEBPR train (**Table 3.3**). In addition, the overall release of ARB in membrane effluent of the 25 day SRT train (**Table 3.2**) was found to be considerably higher than that for the 60 day SRT system and this may have been caused by integrity breaches in membranes and attached bacterial growth in the effluent discharge piping. Due to the importance of these two pathways (foam and membrane-treated effluent) in ARB discharge and the lack of knowledge on the microbial community associated with each of them, the first part of this section is focused on studying the taxonomic composition of bacteria (including both the culturable and unculturable microbiomes) in a MEBPR system.

To address this research question, the compositions of groups of abundant (relative abundance > 1%), intermediate (0.1% < relative abundance < 1%), and rare taxa (relative abundance < 0.1%) in influent, mixed liquor, foam and membrane-treated effluent of a MEBPR train operating at SRT = 25 days were compared. Comparison of the relative abundances of foam microorganisms and underlying mixed liquor bacterial communities was also of interest to determine whether excess growth of filamentous bacteria could be one of the causes of foam formation in the MEBPR process. Profiling the taxonomic composition of bacteria in influent and different compartments of the MEBPR process could also be used to understand the shifts in microbial communities of influent relative to mixed liquor and examine if a selectivity in the community of membrane-treated effluent bacteria is observed, compared to the mixed liquor.

It was previously described in Section 1.2 that, despite the large number of studies which have focused on identification of ARGs in activated sludge, effluent discharge and waste-derived products such as biosolids (Zhang and Zhang, 2011; Szczepanowski et al., 2009; Munir et al., 2011; Gatica and Cytryn, 2013), the diversity of resistance determinants in foam formed in MEBPR systems has remained unexplored. Hence, the second part of this section presents results using both PCR-based and functional metagnomics approaches to assess some ARGs present in the MEBPR-formed foam at SRT = 25 days. Combined with observations in Section 3.2 on the percentage of ARB in foam, this section highlights the potential role of disposed foam in distribution of antimicrobial resistance (AR) to the

environment.

3.3.1 Profiling the Taxonomic Composition of Bacterial Communities in Influent and the MEBPR Environments

In order to identify the taxonomic structure of bacterial communities in the influent and the different compartments of the MEBPR treatment process, 16S rRNA gene V4 amplicons corresponding to the total genomic DNA (including DNA sources from both culturable and unculturable cells) of influent, mixed liquor (anaerobic, anoxic and aerobic reactors), foam and membrane-treated effluent in a MEBPR train operating at 25 day SRT were sequenced on an Illumina MiSeq platform. The procedure for extraction of the genomic environmental DNA was described in Section 2.4.2.1. Section 2.4.2.4 provided the details of the sample preparation procedure including the pooling of DNA samples collected on December 2014, January and February 2015 as well as PCR, sequencing and partial analysis of data.

In the present project, the taxonomic profiles of bacterial communities in influent and the MEBPR process with abundant (relative abundance > 1%), intermediate (0.1% < relative abundance < 1%) and rare (relative abundance < 0.1%) operational taxonomic units (OTUs) were analyzed using three approaches:

(1) Measuring the distance between samples by hierarchical cluster analysis
(2) Analysis of the richness and evenness of bacterial communities by measuring the Shannon-Wiener diversity index
(3) Comparison of the relative abundances of taxa using bubble plots

Using the table of OTUs as input data in the pvclust package, hierarchical cluster analysis in the present project was performed in R. Detailed description of the methods applied is provided in Section 2.4.2.4. As the hierarchical clustering algorithm calculates the distance matrix between samples, it can be used to describe the influence of the wastewater treatment process as well as membrane filtration on bacterial community assemblages obtained from short-read Illumina Miseq sequencing data. A cluster dendrogram of abundant taxa with relative abundance > 1% corresponding to the four analyzed environments is shown in **Figure 3.25**.

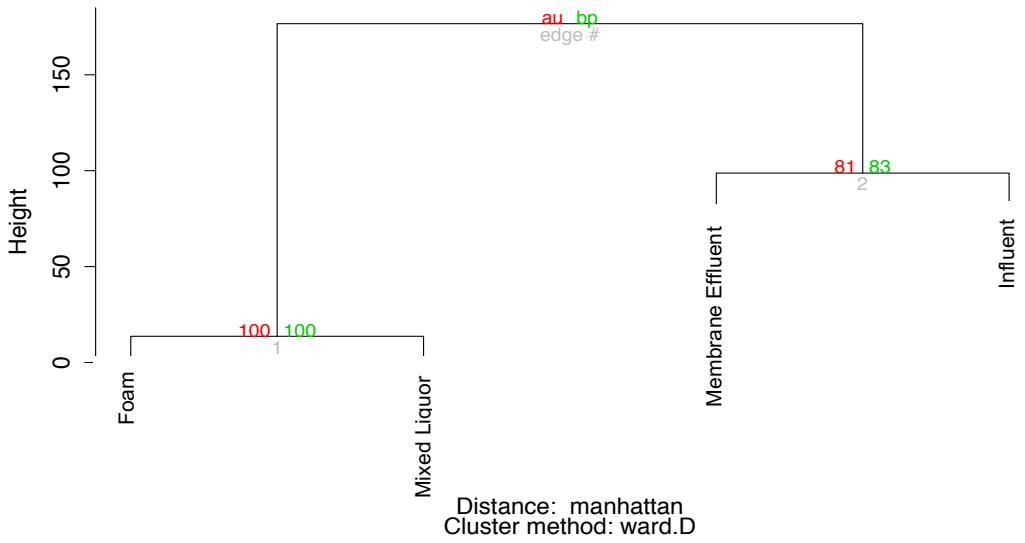


Figure 3.25 Cluster dendrogram of taxa with relative abundance > 1% in influent and MEBPR environments (SRT = 25 days)

In **Figure 3.25**, the vertical axis of the dendrogram represents the dissimilarity between clusters and the horizontal axis is representative of the samples (i.e. influent, mixed liquor, etc). Each linkage of two clusters is represented by a horizontal line whose vertical position indicates the distance between clusters. Observations from the clustering analysis shows that foam and mixed liquor were similarly clustered indicating that these environments were more similar to each other than they were to influent and treated effluent (**Figure 3.25**). This could imply a shift in the bacterial communities from influent to mixed liquor in the MEBPR process and then to the membrane-treated effluent.

In addition, it can be seen that the connection between foam and mixed liquor was the closer link to the bottom of the dendrogram compared to that of the influent and membrane-treated effluent, which confirms a smaller distance between them (**Figure 3.25**). As the approximately unbiased (au) *p*-value of foam and mixed liquor was higher than 95%, these clusters were considered to be strongly supported by data. The similarity in the taxonomic composition of foam and anoxic mixed liquor has been previously reported by Hall and

coworkers (2011) who compared the properties of foam and mixed liquor in the anoxic zone of MEBPR processes (12 and 20 day SRTs) at the UBC pilot plant. Not only did they observe similar contents of nitrogen (N), phosphorus (P) and organic matter in the two environments, but also they found approximately 80% similarity between the community of foam and mixed liquor bacteria by ribosomal intergenic spacer analysis (RISA).

Figure 3.26 provides a graphical view of the Shannon diversity index for influent and the MEBPR environments (detailed methods applied were provided in Section 2.4.2.4). Shannon diversity index considers both the richness and evenness of species in microbial communities using the OTU table imported as a matrix. Briefly, this index is calculated by multiplying the relative abundance of each species by its log (natural or other bases), repeating this step for all different species and finally, computing the negative sum of these numbers. As observed in **Figure 3.26**, Shannon diversity measures were highest in foam and mixed liquor compared to influent and effluent, implying that foam and mixed liquor bacterial communities possessed more evenly distributed and diverse taxa, compared to those of the influent and membrane-treated effluent.

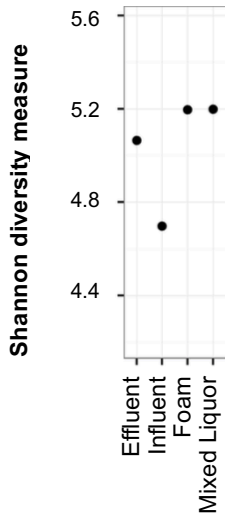
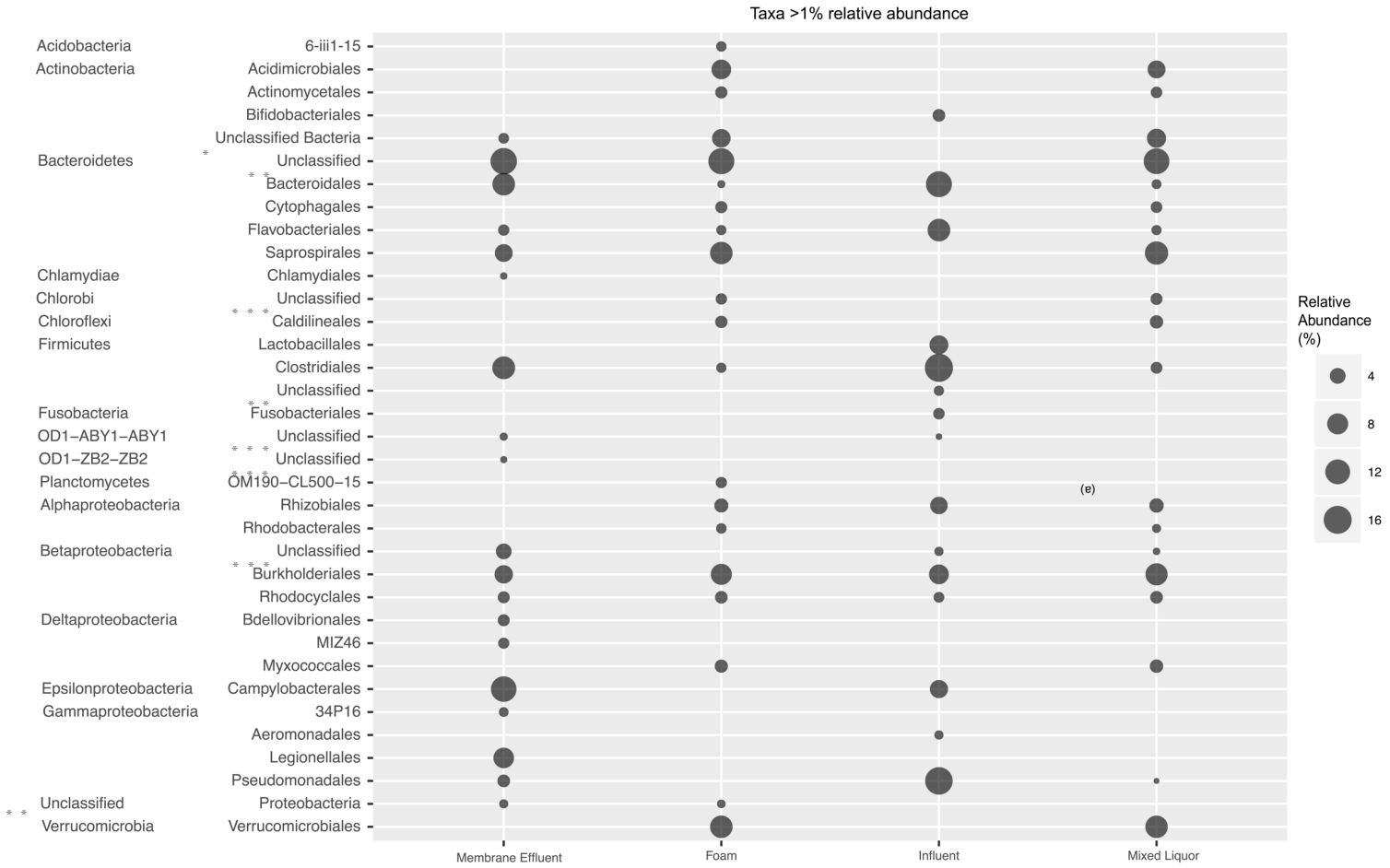


Figure 3.26 Comparison of Shannon index between influent and MEBPR bacterial communities

The bubble plots corresponding to the taxa with relative abundances > 1% in the studied environments is shown in **Figure 3.27**. In order to prepare the bubble plots, the following steps were taken.

1. The table of OTUs (provided by the Microbiome Insights Inc. at the genus level) was re-classified at the order level and the sum of the number of reads belonging to each unique taxa at the order level was calculated. In total, 102, 79, 111 and 114 unique OTUs were obatained for downstream analysis of the membrane-treated effluent, influent, foam and mixed liquor bacterial communities at the order level, respectively.
2. The relative abundance of taxa was determined by dividing the read counts of unique taxonomies to the total number of reads produced for each sample, expressed as percentage.
3. Taxa in each environment (e.g. influent, mixed liquor, etc) were then ordered from the highest relative abundance to the lowest and separated into three groups of abundant, intermediate and rare OTUs.
4. Once taxa were ordered by relative abundance, they were re-ordered by "environment" so that there existed three final lists by abundance for each sample.
5. Bubble plots were made in R using the table corresponding to the relative abundance data at the order level (classified by groups) as an input in the ggplot2 package.



*: Obtained sequences couldnot be assigned to existing taxa at the phylum level
**: Obtained sequences couldnot be assigned to existing taxa at the class level
***: Obtained sequences couldnot be assigned to existing taxa at the order level

Figure 3.27 Taxonomic composition of OTUs with relative abundance > 1% in influent and MEBPR Environments (SRT = 25 days)

As observed in **Figure 3.27**, the influent, mixed liquor, foam and membrane-treated effluent environments exhibited a total of 15, 19, 20 and 19 bacterial orders with relative abundances of more than 1%, respectively. Briefly, bacteria affiliated with the orders *Bacteroidales*, *Flavobacteriales*, *Clostridiales*, *Burkholderiales* and *Rhodocyclales* were similarly detected in all samples (**Figure 3.27**). Contrary to the taxonomic composition of bacteria in mixed liquor, foam and treated effluent, bacteria affiliated with the orders *Bifidobacteriales* (relative abundance in influent: 2.41%), *Lactobacillales* (6.20%), *Fusobacteriales* (1.96%), *Campylobacteriales* (5.60%), *Aeromonadales* (1.35%) and *Pseudomonadales* (15.32%) were considered as abundant data (relative abundance > 1%) in influent. It was also observed that bacteria affiliated with the orders *Acidimicrobiales* (relative abundance in mixed liquor: 5.44%), *Actinomycetales* (1.97%), *Cytophagales* (2.02%), *Saprospirales* (10.29%), *Caldilineales* (2.68%), *Myxococcales* (2.76%), *Verrucomcrobiales* (9.43%) and the phyla *Chloroflexi* (3.92%) and *Chlorobi* (2.13%) were enriched in the MEBPR treatment system.

Consistent with observations in **Figures 3.25** and **3.26**, the taxonomic composition of the foam bacterial community was also found to be most closely related to the mixed liquor microbiome. In general, the phylum *Bacteroidetes* and bacterial orders *Verrucomicrobiales*, *Saprospirales*, *Burkholderiales* and *Acidimicrobiales* were the most abundant taxa in foam and mixed liquor (**Figure 3.27, Appendix C** (**Figures C.3** and **C.4**)).

Lawson (2014) studied the taxonomic composition of bacterial communities in the redox reactors (anaerobic, anoxic, aerobic mixed liquor) of the UBC pilot plant MEBPR system operating at a 15 day SRT using pyrotag sequencing of the small subunit ribosomal RNA gene approach. **Table 3.6** illustrates the comparison between the current study and the relative abundances of major phyla and/or classes of bacteria previously obtained from the Lawson (2014) study period.

Table 3.6 Comparison of the relative abundance of bacterial phyla/classes in influent and MEBPR environments between the present study and Lawson (2014) study

Phyla/Class	Relative Abundance (%) (this study) (Illumina MiSeq approach)				Relative Abundance in mixed liquor (%) using Pyrotag community analysis (Lawson, 2014)
	Influent	Mixed liquor	Foam	Membrane effluent	
Alpha-proteobacteria	5.61	6.55	6.96	1.97	5.1
Beta-proteobacteria	10.56	13.06	11.89	12.08	14.8
Delta-proteobacteria	0.66	3.71	3.59	5.04	3.8
Gamma-proteobacteria	23.26	3.08	2.43	13.03	2.7
Epsilon-proteobacteria	5.60	0.58	0.58	12.86	not measured
Actinobacteria	3.70	7.92	10.43	1.21	32.0
Acidobacteria	0.13	1.35	1.97	0.18	0.7
Bacteroidetes	23.00	28.42	27.77	30.83	25.0
Chroloflexi	0.14	3.92	3.72	0.10	1.9
Cyanobacteria	0.01	0.01	0.03	0.19	0.2
Firmicutes	24.37	3.24	2.58	11.01	2.8
Nitrospirae	0.11	0.89	0.80	0.15	0.8
Planctomycetes	0.10	2.83	4.02	0.17	3.5
Verrucomicrobia	0.53	10.41	10.46	1.60	2.6
Others	2.23	14.47	13.13	9.57	4.1

From **Table 3.6**, it can be seen that, with the exception of quantitative differences in the relative abundances of bacteria affiliated with the phyla *Verrucomicrobia* (2.6% in Lawson (2014) study vs 10.41% in the present study) and *Actinobacteria* (32% in Lawson (2014) study vs 7.92% in the present study), the mixed liquor bacterial community structure at a 15 day SRT (obtained from pyrosequencing data in Lawson (2014) study) and a 25 day SRT (the present study) were comparable. Since these two studies did not profile the taxonomic composition of MEBPR bacterial communities under identical influent input and strict parallel

operating conditions, the moderately extended SRT operation (25 days) cannot be proposed as the only cause of the considerable increase in the relative abundance of bacteria affiliated with the phylum *Verrucomicrobia* and the reduction of bacteria affiliated with the phylum *Actinobacteria*. Another variable that makes interpretation of the role of SRT in the composition of bacterial communities even more complicated is the use of different sequencing approaches in these studies. Under and over-representation of bacterial communities due to the application of different sequencing techniques have been previously reported in the literature (Claesson et al., 2010; Nelson et al., 2014). In one example, Albertsen et al. (2012) reported considerable differences in the relative abundance of *Actinobacteria* and *Chloroflexi* using quantitative FISH assays and a metagenomic sequencing approach. Despite recent advances to investigate the taxonomic structure of bacterial communities in EBPR systems (Martín et al., 2006; Kong et al., 2007; Wong et al., 2005, Albertsen et al., 2012), further attention is still required to study the role of SRT on the composition of bacterial communities in these treatment settings.

Consistent with the observations from **Figure 3.27**, **Table 3.6** indicates a shift in the community of bacteria from influent to MEBPR mixed liquor. As an example, while a considerable reduction was observed in the relative abundance of bacteria affiliated with the phylum *Firmicutes* (3.24% in mixed liquor vs 24.37% in influent) and the classes G*amma-proteobacteria* (3.08% vs 23.26%) *and Epsilon-proteobacteria* (0.58% vs 5.60%) in mixed liquor, the relative abundance of mixed liquor bacteria affiliated with the phylums *Actinobactaria* (3.70% in influent vs 7.92% in mixed liquor)*, Bacteroidetes* (23% vs 28.42%), *Verrucomicrobia* (0.53% vs 10.41%), *Planctinoycetes* (0.1% vs 2.83%), *Nitrospiraea* (0.11% vs 0.89%), *Chloroflexi* (0.14% vs 3.92%), *Acidobacteria* (0.13% vs 1.35%) and the classes *Delta-proteobacteria* (0.66% vs 3.71%)*, Beta-proteobacteria* (10.56% vs 13.06%) and *Alpha-proteobacteria* (5.61% vs 6.55%) increased relative to the influent. In most of the phyla or classes of bacteria where increases in relative abundances of taxa were observed, there is at least one genus of bacteria which is known to mediate key biological processes in EBPR systems including fermentation, nitrification, denitrification, hydrolysis and fermentation. As an example, bacteria affiliated with the class *Nitrospira* (phylum *Nitrospiraea*) are known to be involved in nitrification processes and bacteria affiliated with the genus *Thauera* (class *Beta-proteobacteria*) belong to the group of denitrifiers.

Despite observable differences in the composition of wastewater input, system configurations and operating conditions, literature studies have reported identical or closely related core bacterial communities in EBPR systems (Nielsen et al., 2010; Oehmen et al., 2007). **Table 3.7** summarizes the relative abundances of core bacterial communities in mixed liquor and foam in the 25 day SRT MEBPR train in the present study. The list of taxa involved in EBPR processes (including hydrolysis, fermentation, nitrification, denitrification and biological P removal) is based on taxa identified in Lawson (2014) and Nielsen et al. (2010) studies.

Table 3.7 indicates that many taxa involved in the biological wastewater treatment processes of the UBC pilot plant 25 day SRT MEBPR train belonged to the groups of intermediate (relative abundance between 1% and 0.1%) and rare (relative abundance less than 0.1%) OTUs. In general, observations from the present study showed that the relative abundance of more than 51% of OTUs in mixed liquor was less than 0.1%. Consistent with these observations on finding the majority of OTUs in the group of rare taxa, Lawson (2014) showed that many rare OTUs in the redox zones of the UBC MEBPR system (SRT = 15 days) maintained a high level of activity. The taxonomic composition of intermediate and rare OTUs as well as dendrograms corresponding to influent, mixed liquor, foam and membrane-treated effluent sequencing data are compared in **Figures C.1** to **C.4 (Appendix C)**.

The considerable similarity in relative abundance of mixed liquor and foam bacterial communities showed that foam disposal did not cause any major loss of major functional groups from the MEBPR system operating at 25 day SRT (**Table 3.7**). In addition, it was observed that foam formation in the MEBPR train was not caused by the excess growth of common foam formers such as *Nocardia spp.*, *Microthrix parvicella and Actinomycetes.* This observation is consistent with previous work by Cosenza et al. (2013), who reported a negligible abundance of common filamentous bacteria in foam of a MBR wastewater treatment pilot plant in Italy. Cosenza and coworkers (2013) introduced extracellular polymeric substances (EPSs) as one of the factors involved in fouling and foaming phenomena in MBRs. The importance of EPSs in foaming in MBRs has been previously reported by Di Bella et al. (2011) and You and Sue (2009).

Table 3.7 Relative abundances of core bacterial communities involved in biological treatment processes in mixed liquor and foam in the present study (SRT = 25 days)

Functional group	Taxa (higher classification)	Relative abundance (%)		Functional group	Taxa (higher classification)	Relative abundance (%)	
		Mixed liquor	Foam			Mixed liquor	Foam
Nitrifiers/ Denitrifiers	Class *Nitrospira*	0.89	0.80	Filamentous Bacteria	Phylum *Chloroflexi*	3.92	3.72
	Family *Hyphomicrobiaceae* (*Alpha-proteobacteria*)	0.84	0.76		Genus *Gordonia* (*Actinobacteria*)	0.14	0.36
	Genus *Rhodobacter* (*Alpha-proteobacteria*)	0.42	0.45		Genus Nocardia (*Actinobacteria*)	0.02	0.02
	Family *Rhodocyclaceae* (*Beta-proteobacteria*)	2.39	2.41	Hydrolyzers	Phylum *Chloroflexi*	3.92	3.72
	Genus *Dechloromonas* (*Beta-proteobacteria*)	0.09	0.17		Class *Anaerolineae*	3.51	3.26
	Genus *Zoogloea* (*Beta-proteobacteria*)	0.05	0.03		Family *Caldilineaceae* (*Caldilineae*)	2.68	2.29
	Genus *Thauera* (*Beta-proteobacteria*)	0.03	0.01		Class *Thermomicrobia*	0.06	0.14
	Family *Comamonadaceae* (*Beta-proteobacteria*)	9.10	8.03		Order *Cytophagales (Cytophagia)*	2.02	2.18
	Family *Nitrosomonadaceae* (*Beta-proteobacteria*)	0	0.04		Order *Sphingobacteriales* (*Sphingobacteria*)	0.35	0.29
Fermenters	Genus *Lactococcus* (*Bacilli*)	0.13	0.10		Family *Chitinophagaceae* (*Chitinophaga*)	0.17	0.22
	Genus *Streptococcus* (*Bacilli*)	0.66	0.63		Order *Flavobacteriales* (*Flavobacteria*)	1.55	1.55
	Family *Lachnospiraceae* (*Clostridia*)	0.10	0.05		Genus *Chryseobacterium* (*Flavobacteria*)	0.01	0.01
	Family *Christensenellaceae* (*Clostridia*)	0.10	0.22		Genus *Gordonia* (*Actinobacteria*)	0.14	0.36
	Family *Ruminococcaceae* (*Clostridia*)	0.54	0.43		Genus *Mycobacterium* (*Actinobacteria*)	0.74	0.64
	Genus *Paludibacter* (*Bacteroidetes*)	0.65	0.43	PAOs	Genus *Propionivibrio* (*Beta-propoteobacteria*)	0.34	0.26
	Oder *Bacteroidales* (*Bacteroidetes*)	1.49	1.15		Family *Candidatus-Accumlibacter* (*Beta-proteobacteria*)	0	0.01

As can be seen in **Figures 3.27, C.3**, **C.4** and **Table 3.6**, the community of membrane-treated effluent bacteria exhibited reductions in the relative abundances of some bacterial

groups, such as bacteria affiliated with the classes *Actinobacteria* (7.92% in mixed liquor vs 1.21% in treated effluent), *Alpha-proteobacteria* (6.55% vs 1.97%), *Verrucomicrobia* (10.41% vs 1.60%) and the phyla *Chlorobi* (2.13% vs 0.05%), *Chloroflexi* (3.92% vs 0.1%) and *Nitrospirae* (0.89% vs 0.15%) compared to those of the mixed liquor. In addition, increases were observed in the relative abundances of bacteria affiliated with the classes *Gamma-proteobacteria* (3.08% in mixed liquor vs 13.03% in treated effluent), *Epsilanobacteria* (0.58% vs 12.86%), *Firmicutes* (3.24% vs 11.01%) and the order *Legionellales* (0.09% vs 7.63%) in the community of membrane-treated effluent bacteria compared to the mixed liquor bacterial composition.

The differences between the taxonomic composition of effluent and mixed liquor bacteria could be due to various parameters such as selective microbial adhesion and biofilm formation on the membrane surface and bacterial release due to membrane integrity breaches as well as selective bacterial regrowth inside the effluent discharge tubing after filtration. More comprehensive studies are required to investigate the role of both membrane aging, as well as bacterial attached growth in the interior surface of discharge lines, on the composition and structure of membrane-treated effluent bacterial communities.

It should be remembered that in the present study, the relative abundance of taxa in each community was calculated by dividing the read counts of unique taxonomies by the total read counts and the absolute concentration (distribution) of taxa (per sample volume) was not studied. However, considering the high efficiency of membranes in the removal of total cultivable heterotrophic bacteria (**Table 3.2**), it is expected that all bacterial groups in mixed liquor (including the bacterial orders which exhibited an increase in their relative abundance in membrane-treated effluent) were decreased in their absolute concentration by orders of magnitude by passage through membrane pores.

One of the major disadvantages of culture-dependent techniques is that the total culturable heterotrophic bacteria does not represent the total environmental bacterial community (Gensberger et al., 2015). Stefani and coworkers (2015) showed that two methods of isolation and pyrosequencing exhibited significant differences in the composition of bacterial communities identified in hydrocarbon-contaminated soils. As an example, they observed that none of their isolated soil bacteria belonged to the group of major OTUs which was

obtained by pyrosequencing. Similar findings were observed in the present research.

Using the table of OTUs obtained from sequencing data of membrane-treated effluent, the relative abundances of the 16S-sequenced MH- and R2A-isolated families of effluent bacteria (Sections 3.2.5.2 and 3.1.4) were calculated. The results indicated that, except for bacteria affiliated with the families *Comamonadaceae* (relative abundance: 4.9%) and *Moraxecellaceae* (2.4%), the other identified families of bacteria (e.g. *Xanthomonadaceae*, *Pseudomonadaceae*, etc) belonged to the groups of intermediate and rare OTUs. Due to the restricted number of isolated bacteria (n = 56); however, our observations on the limitations of MH and R2A media to culture representative communities of total effluent bacteria are inadequate. Further research is required to investigate the composition of effluent-isolated bacteria using different culturing media and to provide a comparehensive comparison between treated effluent bacterial communities using culture-based and culture-independent technologies.

3.3.2 Identification of ARGs in the Foam Microbiome

As functional metagenomics enable the uncovering of novel resistance determinants, the application of this approach to study the diversity of ARGs in environmental microbiomes such as soil, has gained more attention recently (Donato et al., 2010; Hatosy and Martiny, 2015). Some studies have also focused on the identification of ARGs and associated host microorganisms in the metagenomic libraries constructed from compartments of wastewater treatment processes (Amos et al., 2014; Uyaguari et al., 2011; Li et al., 2015; Parsley et al., 2010; Munck et al., 2015).

In the present research and to address the objective of the project regarding the diversity of antibiotic resistance genes (ARGs) in MEBPR foam, both functional metagenomics approach and PCR-based examination (to amplify the well-characterized ARGs) were applied.

As the very first attempt to detect resistance determinants in foam in the MEBPR system operating at 25 day SRT, a fosmid library was constructed from genomic DNA of a sample of foam collected on August 2014. Details of the procedures for construction, as well as

functional screening of the foam metagenomic library, were described earlier in Section 2.4.2.5. As previously noted, due to time constraints, only about 24,200 infected bacterial cells (with foam DNA) were plated on LB agar plates and screened for resistance.

In order to estimate the average insert size of the library, a set of 10 library clones were randomly selected, and the size of the fosmids was determined by plasmid isolation, enzymatic digestion (BamHI restriction enzyme) followed by agarose gel electrophoresis and a comparison of the bands of the sample with a DNA ladder (**Appendix D**). Using this approach, the average insert size of this partial library was calculated to be 29.2 kb, which represents a total of about 0.67 Gb of foam-cloned DNA.

Screening the foam fosmid library for carbenicillin (CARB) resistance, two $CARB^r$ clones, *E. coli* EPI300:F3.18 and *E. coli* EPI300:N7.49, with insert sizes of about 30.8 and 29 kb (**Appendix D**) were recovered. It should be noted that due to the limited prescription of CARB, it was expected that a low frequency of resistance to this antibiotic in hospital water and WWTPs would be detected. In the foam microbiome, CARB resistance was detected at the frequency of 8.7×10^{-5} (two $CARB^r$ clones out of 23,060 total screened fosmid clones).

Antimicrobial susceptibility profiles of $CARB^r$ clones and the host (*E. coli* EPI300) were determined by disk diffusion assay testing and are presented in **Table 3.8**. As can be observed, both clones were resistant to antibiotics in the penicillin subgroup of β-lactams such as amoxicillin (AMX) and amplicillin (AMP) (**Table 3.8**). In addition, the *E. coli* EPI300:F3.18 clone was found to be resistant to spectinomycin (SPEC) and sulfamethoxazole (SMX). The MIC values of AMX, CARB, SPEC and SMX in *E. coli* EPI300:F3.18, *E. coli* EPI300:N7.49 and the host are compared in **Table 3.9**.

In order to confirm that the resistance phenotypes observed in the *E. coli* EPI300:F3.18 and *E. coli* EPI300:N7.49 were host-independent, the transferability of resistance to AMX, CARB, SPEC and SMX was tested by transformation of isolated fosmids (pF3.18, pN7.49), to a new host (*E. coli* dH10B strain). Antimicrobial susceptibility profiles as well as digestion patterns of fosmids (using BamHI restriction enzyme) in both hosts (*E. coli* EPI300 and *E. coli* dH10B) were found to be identical. Details of the electrotransformation procedure were provided in Section 2.4.2.7.

Table 3.8 Antimicrobial susceptibility profiles of CARB^r clones in the foam fosmid library[a]

Antibiotic (µg/disk)	Antimicrobial class	*E. coli* EPI300 (host)	*E. coli* EPI300:N7.49	*E. coli* EPI300:F3.18	Antibiotic (µg/disk)	Antimicrobial class	*E. coli* EPI300 (host)	*E. coli* EPI300:N7.49	*E. coli* EPI300:F3.18
AMK (30)	Aminoglycosides				CXM (30)	2nd generation Cephalosporins			
GEN (10)					CTT (30)				
KAN (30)					FOX (30)				
NEO (30)					CFM (5)	3rd generation Cephalosporins			
TOB (20)					CPD (10)				
STR (10)		•	•	•	CAZ (30)				
SPC (100)				•	CRO (30)				
AZM (15)	Macrolide				ETP (20)	Carbapenems			
CIP (5)	Quinolones/ Fluoroquinolones				IMP (10)				
LVX (5)					MEM (20)				
NAL (30)					COL (10)	Polypeptide			
DOX (30)	Tetracyclines				NIT (300)	Nitrofuran			
TET (30)					TMP (5)	Pyrimidine	•	•	•
AMX (30)	Penicillins		•	•	CM (30)	-		•	•
AMP (10)			•	•	FOS (200)	-			
CARB (100)			•	•	SXT (25)	-			•

[a] Filled black circles indicate resistance (diameter of the zone of inhibition = 0.6 centimeter); the absence of a circle mark indicates a larger inhibition zone. AMK: Amikacin, GEN: Gentamicin, KAN: Kanamycin, NEO:Neomycin, TM:Tobramycin, STR: Streptomycin, SPC: Spectinomycin, AZM: Azithromycin, CIP: Ciprofloxacin, LVX:Levofloxacin, NAL: Nalidixic acid, DOX: Doxycycline, TET: Tetracycline, AMX: Amoxicillin, AMP: Amplicillin, CARB: Carbenicillin, CXM:Cefuroxime, CTT:Cefotetan, FOX:Cefoxitin, CFM: Cefixime, CPD:Cefpodoxime, CAZ:Ceftazidime, CRO:Ceftriaxone, ETP:Ertapenem, IMP: Imipenem, MEM:Meropenem, COL: Colistin, NIT:Nitrofurantoin, TMP: Trimethoprim, CM: Chloramphenicol, FOS:Fosfomycin, SXT: SMX+TMP

Table 3.9 MIC values of the *E. coli* EPI300 (host strain) and CARB^r clones in the foam fosmid library)

Antibiotics	MIC (µg/mL)		
	E. coli EPI300	*E. coli* EPI300:F3.18	*E. coli* EPI300:N7.49
AMX[a]	3-4	>256	>256
SPEC[a]	3-4	128	not measured
CARB[b]	31.25	>500	>250
SMX[b]	3-4	>250	3-4

[a]: MIC determined by the E-test method, [b]: MIC determined by the standard two-fold serial broth microdilution method

In order to identify ARGs in the EPI300:pF3.18 (CARB^r clone), a TN5 insertion library was constructed. Further sequencing of the SMX-sensitive *E. coli* EPI300:pF3.18:TN5.N3 (Tn5 library clone) using primer KAN-2-FP-1 as well as PCR, confirmed the presence of the *sul*1 gene in the pF3.18 fosmid (PCR reactions and conditions were provided in Section 2.4.2.2). Since class 1 integrons are commonly associated with the presence of the *sul*1 gene in their 3´conserved segment, the presence of the class 1 integron integrase gene (*int*1) was then assessed by PCR-based examination using primers K159 and K160 (**Tables 2.8** and **2.9**). After amplification of the *int*1 gene in pF3.18, the fosmid was sequenced using primers hep58 and hep59 to identify the integron-associated ARGs (Section 2.4.2.2). BLAST analysis of nucleotide sequences corresponding to the class 1 integron variable region showed 100% identity to partial sequences of the *bla*$_{OXA-2}$ and *orf*D genes initially identified by Anderson and coworkers (1965) in the broad-host range IncN R-brighton plasmid (known as plasmid R46) in *Salmonella typhimurium* (Stokes and Hall, 1992). OXA-type β-lactamases (class D oxacillinases) are known to confer resistance to carboxypenicillin (e.g. CARB) and aminopenicillins (e.g. AMX and AMP) (Opazo et al., 2012; Poirel et al., 2010; Strateva and Yordanov, 2009). As R46 integron cassettes (the best hit match to *bla*$_{OXA-2}$ and *orf*D gene sequences in the present study) contains an *aad*A1 gene which confers resistance to streptomycin (STR) and SPEC, PCR was then conducted to examine the presence of this gene in pF3.18. Gel electrophoresis of the amplified PCR products confirmed that aadA1 gene was also present in pF3.18.

To determine whether the class 1 integron in the IncN plasmid R46 is a true representation of the gene cassettes observed in pF3.18, the gaps between the ARGs were sequenced. Detailed sequencing steps are provided in **Figure C.5** (**Appendix C**). The structure of the class 1 integron in pF3.18 is shown in **Figure 3.28**. The first open reading frame (ORF), located immediately downstream of the *int*1 gene, and the third ORF (827 bp DNA fragments) encode a class D β-lactamase which shared 100% identity with the *bla*$_{OXA-2}$ gene. This gene is assisted with the second ORF of the 791 bp DNA fragment which encodes an aminoglycoside adenyltransferase which shared 100% identity with the *aad*A1 gene.

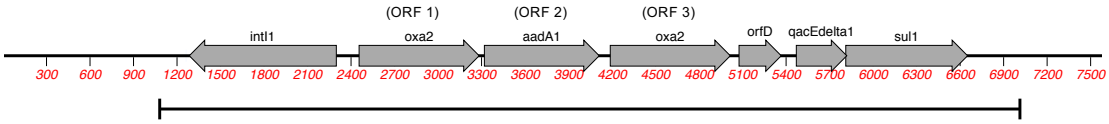


Figure 3.28 Map of the class 1 integron in plasmid R46 (Accession Number: M95287) and the corresponding sequenced region in pF3.18 with 100% identity (black line with perpendicular heads)

It should be noted that the presence of the *aad*A1 gene, bla_{OXA-2} gene, *int*1 gene, as well as the *sul*1 gene in the original foam DNA (used for construction of the foam library), was confirmed by PCR-based examination and gel electrophoresis analysis, the positive control being the purified fosmid pF3.18.

Integrons are one of the major types of mobile genetic elements (MGEs) which are involved in the capture, mobilization, expression and spread of ARGs in bacterial communities (Koczura et al., 2012). Among different classes of integrons, class 1 integrons are the most common among MDR bacteria and play important roles in the distribution of ARGs. Generally, a typical class 1 integron is composed of the integrase gene (*int*1), promoters and a recombination site in the 5′ conserved segment and the *qac*E gene (encoding resistance to quaternary ammonium compounds) and the *sul*1 gene in the 3′ conserved segment and a variable region (Han et al., 2012). The variable region may consist of one or more gene cassettes as can be seen in **Figure 3.28**.

Over the past several years, many studies have shown that effluent discharges from WWTPs increase the frequency of the occurrence of integron-positive strains and various gene cassettes in receiving waters (Guardabassi et al., 2002; Ma et al., 2011; Goñi-Urriza et al., 2000; Reinthaler et al., 2003; Tennstedt et al., 2003; Stalder et al., 2014; Rahube and Yost, 2010; Mokracka et al., 2012; Ma et al., 2013; Zhang et al., 2011). However, detection of ARGs in pF3.18 provides the first evidence of the occurrence of integron-associated ARGs in the foaming compartment of a MEBPR process.

While the occurrence of the *aad*A1 gene, bla_{OXA-2} gene and the *sul*1 gene in activated sludge or final effluent of wastewater treatment plants (WWTPs) has been frequently reported (Simo Tchunite et al., 2016; Szczepanowski et al., 2004a; Anand et al., 2016; Marathe et al., 2013; Szczepanowski et al., 2009), the present study is the second to report that the R46 class 1 integron variable region has been detected in a sample from a wastewater treatment system. The presence of bla_{OXA-2}-*aad*A1-bla_{OXA-2} gene cassette arrays was first reported by Moura and coworkers (2012) in an *Aeromonas caviae* strain isolated from activated sludge of a WWTP in Ermesinde, Portugal.

Further investigation is still required to study the abundance, quantity, as well as the removal efficiency of integrons through disposal of foam bacteria from MEBPR processes. In addition, as comprehensive knowledge of the diversity and function of ARGs is the first step toward proper management of the discharge of ARGs and ARB from the by-products of WWTPs, construction and functional screening of large metagenomic libraries from the various compartments of WWTPs, is of the highest priority.

Due to the importance of MEBPR foam to the overall potential for release of ARB and ARGs, PCR-based examination was performed and the presence of some well-characterized ARGs in the foam genomic DNA (used for construction of the foam fosmid library) was also assessed. A summary of all the existing ARGs in foam is shown in **Table 3.10**.

Table 3.10 Diversity of detected genes in foam DNA (SRT = 25 days)

Antimicrobial Class	Gene name	Encoded enzyme
Sulfonamides	*sul*1[a], *sul*2[a]	dihydropteroate synthetases (DHPS)
β-lactams	bla_{TEM-1}[a]	Class A β-lactamase
	bla_{OXA-2}[b]	Class D β-lactamase
Aminoglycosides	*aad*A1[b]	aminoglycoside adenyltransferase
Quaternary ammonium compounds	*qac*E	small multidrug efflux protein
Tetracyclines	*tet*(G)[a], *tet*(A)[a], *tet*(C)[a]	tetracycline transporters (efflux)
	tet(W)[a]	ribosomal protection protein

[a]: detected by PCR using available primers, [b]: detected using functional metagenomics approach

A summary of conclusions in Section 3.3 includes the following.

- In the present research, the taxonomic compositions of influent, mixed liquor, foam and membrane-treated effluent bacteria in the 25 day SRT train were profiled. Data analysis showed that the majority of mixed liquor taxa involved in biological treatment processes (i.e. nitrification, denitrification, etc) belonged to the groups of intermediate and rare OTUs.
- In addition, hierarchical cluster analysis of bacterial community assemblages (obtained from Illumina Miseq sequencing data) confirmed the significant similarity (small distance) between mixed liquor and foam clusters. A larger diversity and a more even distribution of taxa were also observed in mixed liquor and foam bacterial communities, as shown by the higher values for Shannon diversity indices in these microbiomes compared to influent and membrane-treated effluent bacterial communities.
- Comparison of the taxonomic composition of abundant taxa (relative abundance > 1%) showed that there was a shift in bacterial communities from influent to mixed liquor and then to membrane effluent of the MEBPR train operating at SRT = 25 days.
- PCR-based examination and functional screening of the foam fosmid library revealed the presence of nine ARGs conferring resistance to four classes of antimicrobials (tetracyclines, β-lactams, sulfonamides, aminoglycosides) in the foaming compartment of the MEBPR process.
- Using the functional metagenomics approach, the occurrence of two integron-associated ARGs was reported in the foam microbiome.

4 CONCLUSIONS AND RECOMMENDATIONS

4.1 Summary

The engineering problem that the present project was structured on was the lack of knowledge on the potential pathways of antibiotic resistant bacterial (ARB) release from membrane enhanced biological phosphorus removal (MEBPR) processes operating at long solids retention times (SRTs). The overall objective of the study was to identify factors with critical roles in potential release and spread of antimicrobial resistance (AR) through operation of the MEBPR processes. To satisfy this overall objective, research questions focused on (i) profiling the taxonomic composition of bacterial communities in influent and the 25 day SRT MEBPR train by 16S rRNA gene sequencing of total genomic DNA, (ii) resistance patterns of total cultivable heterotrophic bacteria as well as the *sul*1 gene levels in parallel MEBPR processes, (iii) release of ARB through waste aerobic mixed liquor, foam and membrane-treated effluent discharge, (iv) efficiency of membrane filtration in removal of ARB and multi-drug resistant (MDR) bacteria, (v) the effects of using different media or studying bacterial resistance to different antimicrobials in performance assessment, (vi) diversity of released cultivable heterotrophic bacteria from membrane-treated effluent, (vii) diversity of ARGs in MEBPR foam.

(i): Profiling the Taxonomic Composition of Bacterial Communities in Influent and the 25 Day SRT MEBPR Train by 16S rRNA Gene Sequencing of Total Genomic DNA

In the present project, the microbiomes of influent wastewater and the three internal redox compartments of the 25 day SRT MEBPR train (mixed liquor, foam and membrane-treated effluent) were compared by analysis of the 16S rRNA gene V4 amplicons using an Illumina Miseq platform. A cluster dendrogram of taxa with relative abundance > 1% confirmed that foam and mixed liquor were similarly clustered and there was a smaller distance between them compared to that between the influent and treated effluent clusters. Comparing the Shannon indices of taxa showed that these two environments (foam and mixed liquor microbiomes) also exhibited greater diversity and a more even distribution of taxa.

Analysis of the taxonomic composition of bacterial communities in the form of bubble plots showed that the most abundant taxa (relative abundance > 1%) in foam and mixed liquor belonged to the groups of bacteria affiliated with the phylum *Bacteroidetes* and the orders *Verrucomicrobiales*, *Saprospirales*, *Burkholderiales* and *Acidimicrobiales*.

In addition, the presence of bacteria affiliated with the orders *Bacteroidales*, *Flavobacteriales*, *Clostridiales*, *Burkholderiales* and *Rhodocyclales* as the common taxa with relative abundance > 1% was also confirmed in all four microbiomes. However, a shift in the taxonomic composition of bacteria with abundant operational taxonomic units (OTUs) was observed from influent to mixed liquor. As an example, it was shown that the 25 day SRT MEBPR train facilitated the growth of bacteria affiliated with the phyla *Actinobactaria, Bacteroidetes*, *Verrucomicrobia*, *Planctinoycetes, Nitrospiraea, Chloroflexi, Acidobacteria* and the classes *Delta-proteobacteria, Beta-proteobacteria* and *Alpha-proteobacteria*.

Data also showed that the taxonomic composition of the membrane-treated effluent bacterial community exhibited major differences to that of the mixed liquor. As an example, it was observed that while the relative abundance of bacteria affiliated with the phylum *Verrucomicrobia* was reduced from 10.4% in mixed liquor to 1.6% in treated effluent, the relative abundance of bacteria affiliated with the class *Gamma-proteobacteria* was about 10% higher in treated effluent (3.08% in mixed liquor vs 13.03% in effluent). Profiling the taxonomic composition of bacteria in mixed liquor also showed that the majority of taxa involved in the engineered biological processes belonged to the groups of intermediate (relative abundance between 1% and 0.1%) and rare (relative abundance less than 0.1%) OTUs. Finally, it was observed that the relative abundance of more than half of the OTUs in mixed liquor belonged to the group of rare MEBPR taxa.

(ii): Resistance Patterns of Total Cultivable Heterotrophic Bacteria as well as the *sul*1 Gene Levels in Parallel MEBPR Processes

The present research was conducted as a comparison between parallel MEBPR processes operating at 25 (control) and 60 day SRTs, during the period of December 2014 to February

2015 (Section 3.2). To ensure broad representation of ARB, more than 4,200 MH- and 3,100 R2A-grown cultivable heterotrophic bacteria from influent, anoxic mixed liquor, foam and membrane-treated effluent were isolated and screened for resistance to sulfamethoxazole (SMX) (50 µg/mL), trimethoprim (TMP) (5 µg/mL), amoxicillin (AMX) (32 µg/mL), tetracycline (TET) (10 µg/mL) and ciprofloxacin (CIP) (5 µg/mL) by growth in liquid medium. Comparison of the ARB ratios showed that, while the abundances of AMX^r, SMX^r and TMP^r heterotrophic bacteria were highest in influent and in the parallel MEBPR environments, the communities of CIP^r and TET^r bacteria were associated with the lowest abundance of resistance.

Comparing the ARB proportions in influent and mixed liquor in parallel MEBPR systems showed that, while the percentage of SMX^r and TMP^r MH and R2A bacteria significantly ($P \leq 0.05$ or $P \leq 0.1$) increased from influent to mixed liquor at the 25 day SRT, the 60 day SRT operation contributed to statistically significant increases ($P \leq 0.05$) in the percentages of ARB from influent to mixed liquors for almost all tested antimicrobials (except for CIP^r bacteria) in R2A bacterial communities.

Statistically significant increases ($P \leq 0.05$ or $P \leq 0.1$) were also observed in the communities of TMP^r, AMX^r, TET^r R2A bacteria from influent to membrane-treated effluent in parallel trains operating at either the 25 or 60 day SRTs. Similar findings were observed in Section 3.1 where the percentage of SMX^r bacteria was compared between influent and membrane-treated effluent heterotrophic communites. In addition, the ARB proportions of mixed liquor and treated effluent bacteria were not statistically different at the 25 day SRT, for any of the tested antimicrobials.

It was also observed that, unlike the 25 day SRT train where the ratios of ARB in foam and underlying anoxic mixed liquor MH and R2A heterotrophic bacteria were not statistically different, the percentages of SMX^r ($P \leq 0.1$), TMP^r ($P \leq 0.05$) and TET^r ($P \leq 0.1$) bacteria exhibited statistically significant elevations in foam in the R2A population at SRT = 60 days, compared to those of the mixed liquor.

Comparing the ARB ratios of mixed liquor bacteria at 25 and 60 day SRTs also showed that, while MH mixed liquor populations did not exhibit any significant differences in ARB ratios between the control (SRT = 25 days) and extended SRT operation (SRT = 60 days), the

ratios of SMX^r R2A bacteria were significantly higher ($P \leq 0.1$) at the 60 day SRT, than those at the SRT = 25 days.

Similar increasing trends of ARB ratios through the MEBPR treatment process were also observed for the abundance of MDR bacteria in MEBPR environments. As an example, it was found that both MH and R2A heterotrophic bacterial communities in mixed liquor and membrane-treated effluent bacteria exhibited greater populations of MDR bacteria, compared to those of the influent. Monitoring the role of SRT variations, the communities of MDR MH and R2A heterotrophs were found to be largest in membrane-treated effluent at the 60 day SRT.

Quantifying the levels of the *sul*1 gene in parallel MEBPR trains (Section 3.1) confirmed a significant increase ($P \leq 0.05$) in the relative abundance of the *sul*1 gene in mixed liquors at 25 and 60 day SRTs, compared to those of the influent. It was also observed that the MEBPR redox zones acted as homogenizing reactors with insignificant differences in their relative concentrations (normalized to the 16S rRNA gene) of the *sul*1 gene. In addition, while the relative concentrations of the *sul*1 gene in membrane-treated effluent at 25 and 60 day SRTs were found not to be significantly higher than those of the influent, the absolute concentrations of this gene (normalized to the original sample volume) were significantly higher in the influent than those of the membrane-treated effluents.

(iii): Release of ARB through Waste Aerobic Mixed Liquor, Foam and Effluent Discharge

Estimating the release rates of ARB through waste aerobic zone mixed liquor, anoxic zone foam, and membrane-treated effluent showed that SMX^r, AMX^r, and TMP^r bacteria were three major types of ARB that were discharged from the parallel MEBPR systems. Due to the combined effects of the considerable difference between the contribution of foam and aerobic mixed liquor in the wastage of solids from the parallel MEBPR systems, as well as the insignificant difference in ARB ratios between foam and mixed liquor for some of the tested antimicrobials, the major vector for the release of ARB was found to be different at the 25 and 60 day SRT conditions. In the 25 day SRT train, the wasted aerobic mixed liquor

exhibited the maximum contribution and in the 60 day SRT, foam wasting was shown to be the major pathway to ARB discharge; this represents the highest priority for further research.

It was also observed that compared to membrane-treated effluent, the combination of waste aerobic mixed liquor and foam was always the main contributor to the release of ARB from parallel wastewater treatment processes. As an example, the average number of TET^r MH bacteria discharged through waste aerobic mixed liquor at the 25 day SRT was about 168 times greater than the counts of TET^r bacteria released in membrane-treated effluent.

In addition, compared to the control train (SRT= 25 days), observations from the present project showed that extended SRT operations (SRT = 60 days) did not considerably change the average fraction of contribution of combined foam and waste aerobic mixed liquor in the release of ARB for some of the tested antimicrobials (AMX, SMX, TET, TMP in MH community and AMX, SMX and TET in R2A bacteria) (**Table 3.3**).

It was also observed that the total release rates of ARB (combination of waste aerobic mixed liquor, foam and membrane-treated effluent) were always lower than the inflow rate (IR) in either MH and R2A media for both SRT conditions. This implies that 25 or 60 days of solids retention time (SRT) could be proposed as optimal operating conditions of the MEBPR systems to reduce the release of cultivable MH and R2A heterotrophic bacteria compared to the influent.

(iv): Efficiency of Membrane Filtration in Removal of ARB and MDR bacteria

Comparing the counts of influent and membrane-treated effluent bacteria in the present project showed that with the exception of TET^r bacteria, the MEBPR processes achieved at least three log reductions of total heterotrophic bacteria and ARB at either a 25 or 60 day SRT. As a result, treated effluent collected after passage through membrane pores was generally found to make a negligible contribution to the spread of ARB from the parallel MEBPR systems. Similar observations were made by comparing the concentrations of the *sul*1 gene (normalized to sample volume) between influent and treated effluent samples

which confirmed membrane-treated effluent as a minor pathway in the release of the *sul*1 gene to the environment.

It was also observed that membrane imperfections (detected on February 2015) dramatically decreased the efficiency of membrane filtration in ARB removal in the 25 day SRT MEBPR train. This caused considerable increases in the average release rates of ARB in effluent from this train, compared to that of the 60 day SRT system.

Despite the overall high efficiency of membranes in the removal of ARB and the concentration of the *sul*1 gene (normalized to sample volume), significant increases were also observed in ratios of SMX and TMP as well as TMP, AMX and TET resistance from influent to membrane effluents during the December 2014 to February 2015 study period in MH and R2A populations, respectively. Similarly, monitoring the MDR patterns of heterotrophic bacteria in the dual trains of the UBC pilot plant showed that the percentage of MDR bacteria increased from influent to treated effluent. In addition, it was observed that effluent total cultivable heterotrophic bacteria (isolated from either MH or R2A media) exhibited the highest proportion of MDR strains in the 60 day SRT train.

Similar findings were also observed when the ratios of SMX^r heterotrophic bacteria were compared between influent and membrane effluent samples collected on January 2014 from the 25 day SRT train. In order to compare the antimicrobial susceptibility patterns of SMX^r heterotrophic bacteria between influent and membrane effluent samples (25 day SRT train), disk diffusion assay testing was performed. The results showed that the ratio of the co-occurrence of resistance to SMX and most of the tested antibiotics exhibited an increasing trend from influent to membrane-treated effluent.

(v): The Effects of Using Different Media or Studying Bacterial Resistance to Different Antimicrobials in Performance Assessment

Isolation of influent and MEBPR heterotrophic bacteria from two non-selective non-differential media (MH and R2A) provided the opportunity for comparative studies of the effects of media type on estimated ARB ratios and MDR patterns under parallel conditions

(i.e. sampling points and dates). Observations from the present study confirmed significant differences in the estimated ratios of ARB for some tested antimicrobials in MH and R2A heterotrophic populations. As an example, while the ratios of AMX, SMX and TMP resistance were found to be greater in the MH population, ratios of CIP resistance were not statistically different in influent and MEBPR environments for the R2A and MH populations at either 25 or 60 days of retention time. In addition, unlike resistance ratios of the MH mixed liquor population which did not exhibit any statistically significant differences under different SRT operations, very long SRT operations (SRT = 60 days) significantly increased the proportions of SMX^r bacteria in R2A population compared to that of the 25 day SRT train. Briefly, the present research demonstrated that media type (MH vs R2A) can influence on the overall outcome of the MEBPR performance assessment in the spread of ARB.

Comparative monitoring studies of MH and R2A populations in the present research suggest that media formulation (high-nutrient level medium vs low-nutrient level medium) could influence the bacterial diversity and preferential growth of heterotrophic organisms. As an example, it was observed that treated effluent strains affiliated with the two classes *Flavobacteria* and *Deinococci* were only isolated from R2A medium. In total, compared to MH bacterial isolates, more diverse phyla of environmental bacteria including *Bacteroidetes* and *Deinococcus-Thermos* were identified among R2A isolated strains.

Lactose fermentation testing assays of 96 MH and 96 R2A effluent bacterial isolates from the 25 day SRT train also exhibited a considerable difference in the proportion of lactose fermenting (Lac+) bacteria (39.5% in MH vs 23.9% in R2A) and potential Gram positive (G(+)) bacteria (6.25% in MH vs 26.04% in R2A) between the MH and R2A groups. In addition, it was observed that either groups of Lac+ or Gram negative G(-) non-lactose fermenting (Lac-) R2A bacteria had lower percentages of AMX^r, SMX^r and TMP^r bacteria compared to those of the MH population. The underlying reason could be the formulation of R2A medium to support the growth of a more diverse range of non-pathogenic waterborne bacteria.

In general, the choice of medium for the isolation of cultivable heterotrophic bacteria (MH, R2A, LB, plate count and nutrient agar), the methodology to determine the percentage of

ARB, as well as the bacterial enumeration schemes utilized, including the incubation time and temperature, could explain why there are inconsistent reports in the literature regarding the ratios of ARB for similar antibiotics in samples collected from WWTPs.

In the present project, 33 membrane-treated effluent bacterial strains with different patterns of resistance to the tested antimicrobials (SMX, AMX, CIP, TET and TMP) were identified by 16S rRNA gene sequencing analysis and characterized based on their resistance phenotypes to a set of 16 antimicrobials. The results showed a considerable increase in the community of MDR bacteria (33.3% vs 75.7%) when the susceptibilities to the extra 16 antimicrobials were reported. This implies the importance of selecting a sufficiently large number of antimicrobial classes while evaluating the performance of WWTPs through comparison of the percentage of MDR bacteria between influent and effluent.

(vi): Diversity of Released Cultivable Heterotrophic Bacteria from Membrane-treated Effluent

In total, 16S rRNA gene sequencing analyses of 56 MH- and R2A-isolated strains identified bacteria affiliated with 15 families, *Enterobacteriaceae*, *Aeromonadaceae*, *Comamonadaceae*, *Xanthomonadaceae*, *Shewanellacea*, *Pseudomonadaceae*, *Microbacteriaceae*, *Caulobacteraceae*, *Enterococcaceae*, *Bacillaceae*, *Moraxellaceae*, *Flavobacteriaceae*, *Sphingomonadaceae*, *Staphylococcaceae* and *Deinococcaceae* in membrane-treated effluent of the 25 day SRT MEBPR train. 16S rRNA gene sequencing analysis also confirmed that the community of ARB, as well as MDR bacteria in treated effluent, was not limited to indicators of faecal contamination such as *E. coli*.

Having combined this observation with the fact that most of the MDR bacteria identified in the present section included at least one known species involved in EBPR-specific functional processes such as nitrification, denitrification and P reduction, emphasizes the necessity of monitoring the release rates of total cultivable heterotrophic ARB to provide a more extensive assessment of the performance of MEBPR systems. In the present research, due to the limitation in the number of 16S-sequenced effluent bacteria, comprehensive

comparison of the MH and R2A effluent populations could not be performed. However, due to the high importance of identification of the tested bacterial community (total heterotrophs in the present study) in studies of AR, it is highly suggested that 16S rRNA gene sequencing be performed on representative groups of MH and R2A isolated strains and the diversity as well as the relative abundance of taxa in each group be determined through 16S-sequencing of the total DNA.

(vii): Diversity of ARGs in MEBPR Foam

Metagenomic libraries are powerful tools to identify the genetic diversity of bacterial communities and to characterize the novel ARGs in environmental bacteria. In the present project, antimicrobial functional screening was performed to test for carbenicillin (CARB) resistance in a small foam metagenomic library which represented a total of about 0.67 Gb of cloned DNA. Two $CARB^r$ clones (EPI300:N7.49 and EPI300:F3.18 clones) were found to be resistant to the penicillin subgroup of β-lactams (e.g. AMX and AMP). The *E. coli* EPI300:F3.18 was also observed to be resistant to SMX and SPEC in two classes of sulfonamides and aminoglycosides.

Sequencing of the SMX-sensitive *E. coli* EPI300:pF3.18:TN5.N3 (Tn5 library clone) as well as PCR-based examination revealed the presence of the *sul*1 gene (conferring resistance to SMX), the *int*1 gene as well as the bla_{OXA-2} gene and the *aad*A1 gene, which are known to confer resistance to CARB and SPEC, in the EPI300:F3.18 clone. In addition, BLAST analysis of nucleotide sequences showed that the class 1 integron variable region in pF3.18 had a 100% similarity to the class 1 integron in IncN plasmid R46 which was initially identified by Anderson et al. (1965) in *Salmonella typhimurium*. Although the presence of the plasmid R46 class 1 integron variable region has been previously documented in a WWTP (Moura et al., 2012), the present study is the first in which integron-associated ARGs have been reported in MEBPR foam.

4.2 Project Conclusions

In response to the concerns regarding the spread of ARB and ARGs from MEBPR systems operating at long SRTs, the present project performed a comparative study monitoring the performance of the UBC pilot plant parallel MEBPR trains operating at 25 day (control) and 60 day (extended) SRTs. The following overall conclusions can be derived from evaluating the performance of parallel MEBPR processes in distribution of AR.

1- MEBPR processes operating at 25 and 60 day SRTs significantly increased the relative abundance of the *sul*1 gene from influent to MEBPR mixed liquors. However, long SRT operations (60 days) did not exhibit a significant impact on levels of the *sul*1 gene in mixed liquor, compared to that of the control (SRT = 25 days).

2- Both 25 and 60 day SRT operations increased the percentage of SMX and TMP resistance in mixed liquor MH and R2A heterotrophic bacterial communities relative to the influent. In addition, extended SRT operation significantly ($P \leq 0.1$) increased the percentage of SMX resistance in R2A heterotrophic community of mixed liquor compared to that of the control.

3- Except for the ratio of CIP resistance, the 60 day SRT operation contributed significant increases in the percentages of ARB from influent to mixed liquor for all tested antimicrobials in R2A bacterial communities.

4- The 25 and 60 day SRT operations did not exhibit considerable differences in the fraction of contribution of combined foam and waste aerobic mixed liquor in release of AMX^r, SMX^r and TET^r heterotrophic bacteria.

5- Unlike the control MEBPR train (SRT = 25 days), the long SRT operation exhibited a significant elevation in the ratios of SMX, TMP and TET resistance in foam R2A population compared to those of the mixed liquor.

6- Membrane filtration always achieved more than two log reductions of ARB in parallel MEBPR systems. However, membrane integrity breaches dramatically increased the average release rates of ARB in treated effluent.

7- In total, 15 different bacterial families (*Enterobacteriaceae, Aeromonadaceae, Comamonadaceae, Xanthomonadaceae, Flavobacteriaceae, Bacillaceae, Pseudomonadaceae, Microbacteriaceae, Caulobacteraceae, Moraxellaceae, Enterococcaceae, Shewanellacea, Sphingomonadaceae, Staphylococcaceae* and *Deinococcaceae*) were found in the community of membrane-treated effluent culturable MH and R2A heterotrophic bacteria at the 25 day SRT MEBPR train.

8- The taxonomic compositions of foam and mixed liquor bacterial communities at the 25 day SRT MEBPR train were found to be closely related.

9- Major differences were observed in the microbiomes of influent, mixed liquor and membrane-treated effluent.

10- Using both PCR-based examination and functional metagenomics approach, a set of nine ARGs (*sul*1, *sul*2, $bla_{\text{TEM-1}}$, $bla_{\text{OXA-2}}$, *aad*A1, *tet*(G), *tet*(W), *tet*(A), *tet*(C)) were detected in the foaming compartment of the 25 day SRT MEBPR system.

11- Waste aerobic mixed liquor and foam were the major contributors to the total ARB release in parallel MEBPR trains operating at 25 and 60 day SRTs, respectively.

12- Membrane-treated effluent bacteria made a negligible contribution to the overall release of ARB from the MEBPR dual trains.

13- AMX, SMX and TMP were associated with the highest abundance of resistance in influent and parallel MEBPR systems.

14- A wider diversity of environmental bacteria with generally lower ratios of AMX, SMX and TMP resistance were observed in the R2A-isolated membrane effluent community compared to MH medium.

15- The resistance ratios of ARB in mixed liquor and membrane-treated effluent bacteria were not significantly different at SRT = 25 days.

4.3 Engineering Significance

This research project has made the following contributions in our understanding of the role of MEBPR process in distribution of AR.

1. The present research demonstrated that MEBPR processes operating at 25 and 60 day SRTs could enhance the potential for the growth of ARB. This study introduced foam, waste aerobic mixed liquor and treated effluent discharge as three pathways of potential ARB release from the MEBPR process. Considering foam as an active component of MEBPR systems with similar taxonomic composition to that of the mixed liquor microbiome, the present research demonstrated that foam could be identified as one of the major sources of ARB and ARGs which will require safe disposal of (e.g. in landfills, incineration facilities) or reuse through land application. In cases where MEBPR foam is intended to be utilized for P recovery and land application (struvite fertilizer), observations from the present research emphasize the need for further studies to understand the fate of ARB and ARGs in foam.
2. This research evaluated the role of elevated SRT in the release of ARB in MEBPR processes operated under identical conditions such as wastewater source, reactor volumes and system configurations. The present study demonstrated that the average total daily release rates of ARB (e.g. AMX, SMX, TET) and the fraction of contribution of combined foam and waste aerobic mixed liquor in release of AMX^r, SMX^r and TET^r bacteria were not considerably different between the 25 and 60 day SRT MEBPR trains.
3. Observations from the present research strongly suggest that the indirect biological tests such as bacterial counts be in the list of routine and continuous monitoring parameters of the MEBPR effluent. The present study demonstrated the statistically significant elevation in the percentage of resistance to some of the tested antimicrobials (e.g. TMP) from influent to membrane-treated effluent and therefore,

the application of advanced supplementary techniques to guarantee the quality of membrane-treated effluent prior to discharge is necessary.

4.4 Recommendations for Future Research

This research is the first study which has attempted to compare the ratios of ARB for five antimicrobials from five different classes in MH and R2A heterotrophic bacterial communities. Observations from this project demonstrated how the choice of medium and studied antimicrobial resistance could influence the assessment of the performance of the wastewater treatment system in the distribution of AR. In this respect, the presence of regulatory guidelines with a comprehensive list of microbial water quality parameters to evaluate the performance of WWTPs in distribution of ARB and ARGs is a necessity. These parameters could include (1) the list of antimicrobials and corresponding resistance genes to be tested, (2) the list of sampling locations or hot spots in WWTPs with potential roles in the release of ARB and ARGs and (3) the list of methodologies and technical approaches to be applied. Understanding the role of wastewater treatment processes with respect to AR demands a uniform microbial water quality assessment plan, through which all WWTPs can be monitored and evaluated.

The present research suggested that formulations of non-selective non-differential media (R2A low nutrient and MH high nutrient medium) could affect the culturable heterotrophic bacterial community and corresponding ARB ratios. In this respect, 16S rRNA gene sequencing analysis is recommended as the most effective approach to obtain a deeper understanding of the effects of cultivation media type on the bacterial community structure. It is suggested that for influent and all compartments of the MEBPR process (mixed liquor, foam and membrane-treated effluent), 16S rRNA gene sequence analysis is performed on the extracted DNA of culturable bacterial isolates and the diversity of recovered heterotrophic bacteria is compared between the two media.

Another topic for future research is to investigate the antibiotic resistome of MEBPR environments. More specifically on foam, our knowledge of the diversity of ARGs and their fate after disposal, as well as the frequency of gene transfer in foam, is not yet clear. As an

example, to our knowledge, no studies have attempted to assess the occurrence of MDR pathogens and ARGs in aerosols from foam.

Precipitation of antibiotics, heavy metals and pathogens during struvite formation has been previously documented (Decrey et al., 2011; Basakcilardan et al., 2007). Hence, the potential environmental risks (e.g. release of ARB and ARGs) associated with the usage of foam, as a source for struvite production, also need to be extensively studied.

More investigation is also required to evaluate the potential of ARB growth in effluent distribution lines. A comprehensive study is needed to profile and compare the taxonomic composition and ARB ratios of effluent bacterial communities after membrane filtration, on the membrane surface, inside the distribution lines and at the discharge points.

Bibliography

Aali, R., Nikaeen, M., Khanahmad, H., Hassanzadeh, A. (2014) Monitoring and comparison of antibiotic resistant bacteria and their resistance genes in municipal and hospital wastewaters. *International Journal of Preventive Medicine* **5**(7): 887–94.

Ajao, A.T., Yakubu, S.E., Umoh, V.J., Ameh, J.B. (2013) Bioremediation of refinery wastewater using immobilized *Burkholderia cepacia* and *Corynebacterium* sp and their transconjugants. *Journal of Xenobiotics* **3**(e4): 19-23.

Akiyama, T. & Savin, M.C. (2010) Populations of antibiotic resistant coliform bacteria change rapidly in a wastewater effluent dominated stream. *Sci Total Environ* **408**(24): 6192–6201.

Al-Bahry, S.N., Mahmoud, I.Y., Al-Khaifi, A., Elshafie, A.E., Al-Harthy, A. (2009) Viability of multiple antibiotic resistant bacteria in distribution lines of treated sewage effluent used for irrigation. *Water Sci Tech* **60**(11): 2939–48.

Albertsen, M., Hansen, L.B.S., Saunders, A.M., Nielsen, P.H., Nielsen, K.L. (2012) A metagenome of a full-scale microbial community carrying out enhanced biological phosphorus removal. *Isme j* **6**(6): 1094-1106.

Alexander, J., Knopp, G., Dostch, A., Wieland, A., Schwartz, T. (2016) Ozone treatment of conditioned wastewater selects antibiotic resistance genes, opportunistic bacteria, and induce strong population shifts. *Sci Total Environ* **559**: 103–12.

Allen, M.J., Edberg, S.C., Reasoner, D.J. (2004) Heterotrophic plate count bacteria—what is their significance in drinking water? *International Journal of Food Microbiology* **92**(3): 265-74.

Amador, P.P., Fernandes, R.M., Prudêncio, M.C., Barreto, M.P., Duarte, I.M. (2015) Antibiotic resistance in wastewater: Occurrence and fate of *Enterobacteriaceae* producers of class A and class C β-lactamases. *Environ Sci Health A Tox Hazard Subst Environ Eng* **50**(1): 26–39.

Amanatidou, E., Samiotis, G., Bellos, D., Pekridis, G., Trikoilidou, E. (2015) Net biomass production under complete solids retention in high organic load activated sludge process. *Bioresour Technol* **182**: 193–9.

Amos, G.C., Zhang, L., Hawkey, P.M., Gaze, W.H., Wellington, E.M. (2014) Functional metagenomic analysis reveals rivers are a reservoir for diverse antibiotic resistance genes. *Vet*

Microbiol **171**(3-4): 441–7.

Anand, T., Bera, B., Vaid, R., Barua, S., Riyesh, T., Virmani, N., Hussain, M., Singh, R., Tripathi, B.J. (2016) Abundance of antibiotic resistance genes in environmental bacteriophages. *Gen. Virol* **97**(12): 3458-66.

Anderson, E.S., Datta, N. et al. (1965) Resistance to penicillin and its transfer in *Enterobacteriaceae*. *The Lancet* **285**(3782): 407-9.

Andersson, D.I. & Hughes, D. (2010) Antibiotic resistance and its cost: Is it possible to reverse resistance? *Nature Reviews Microbiology* **8**: 260-71.

Andreasen, K. & Nielsen, P.H. (2000) Growth of Microthrix parvicella in nutrient removal activated sludge plants: Studies of in situ physiology. *Water Research* **34**(5): 1559-69.

Andrews, J.M. (2001) Determination of minimum inhibitory concentrations. *J. Antimicrob. Chemothe* **48** (Suppl.1): 5–16.

Antunes, P., Machado, J., Sousa, J.C., Peixe, L. (2005) Dissemination of sulfonamide resistance genes (*sul*1, *sul*2, and *sul*3) in Portuguese *Salmonella enterica* strains and relation with integrons. *Antimicrobial Agents and Chemotherapy* ***49***(2): 836–9.

Aoi, Y., Shiramasa, Y., Kakimoto, E., Tsuneda, S., Hirata, A., Nagamune, T. (2005) Single-stage autotrophic nitrogen-removal process using a composite matrix immobilizing nitrifying and sulfur-denitrifying bacteria. *Appl Microbiol Biotechnol* **68**: 124–30.

Auerbach, E.A., Seyfried, E.E., McMahon, K.D. (2007) Tetracycline resistance genes in activated sludge wastewater treatment plants. *Water Research* **41**(5): 1143–51.

Bahadoorsingh, P. (2010) Comparison of nitrification activity in membrane and conventional enhanced biological phosphorus removal processes, Ph.D. Dissertation. Department of Civil Engineering, University of British Columbia, Vancouver, Canada.

Balcazar, J.L. (2014) Bacteriophages as vehicles for antibiotic resistance genes in the environment. *PLoS Pathogens* **10**(7): e1004219.

Balkhed, Å.Ö. (2014) Extended-spectrum β-lactamase-producing *Enterobacteriaceae* (antibiotic consumption, detection and resistance epidemiology). Medical Dissertation No. 1394, Department of Clinical Experimental Medicine Faculty of Health Sciences Linköping University Sweden.

Barakat, M.A. (2011) New trends in removing heavy metals from industrial wastewater. *Arabian Journal of Chemistry* **4**(4): 361-77.

Basakcilardan-Kabakci, S., Thompson, A., Cartmell, E., Le Corre, K. (2007) Adsorption and precipitation of tetracycline with struvite. *Water Environment Research* **79**: 2551-6.

Batt, A., Kim, S., Aga, D. (2007) Comparison of the occurrence of antibiotics in four full-scale wastewater treatment plants with varying designs and operations. *Chemosphere* **68**: 428–35.

Beer, M., Stratton, H.M., Griffiths, P.C., Seviour, R.J. (2006) Which are the polyphosphate accumulating organisms in full-scale activated sludge enhanced biological phosphate removal systems in Australia? *J. Appl. Microbiol* **100**: 233–43.

Behroozian, S., Svensson, S.L., Davies, J. (2016) Kisameet clay exhibits potent antibacterial activity against the ESKAPE pathogens. *mBio* **7**(1): e01842–15.

Beier, S., Cramer, C., Mauer, C., Köster, S., Schröder, H.F., Pinnekamp, J. (2012) MBR technology: A promising approach for the (pre-) treatment of hospital wastewater. *Water Sci Technol* **65**(9): 1648–53.

Beier, S., Köster, S., Veltmann, K., Schröder, H., Pinnekamp, J. (2010) Treatment of hospital wastewater effluent by nanofiltration and reverse osmosis. *Water Sci Technol* **61**(7): 1691–8.

Benefield, L.D. & Randall, C.W. (1980) Biological process design for wastewater treatment. *Prentice-Hall, Englewood Cliffs, N.J.*

Bernatová, S., Samek, O., et al. (2013) Following the mechanisms of bacteriostatic versus bactericidal action using roman spectroscopy. *Molecules* **18**: 13188-99.

Bolton, M. (2012) Comparing two remediation alternatives for diesel-contaminated soil in the arctic using life cycle assessment. Masters Thesis. Department of Civil Engineering, Queen's University, Ontario, Canada.

Börjesson, S. (2009) Antibiotic resistance in wastewater (Methicillin resistant *Staphylococcus aureus* (MRSA) and antibiotic resistance genes. Medical Dissertation No. 1128, Department of Clinical Experimental Medicine Faculty of Health Sciences, Linköping University Sweden.

BC Centre for Disease Control (2010) British Columbia Annual Summary of Antibiotics Utilization, DO Bugs Need Drugs?

Britton, A., Koch, F.A., Mavinic, D.S., Adnan, A., Oldham, W.K., Udala B. (2005) Pilot-scale

struvite recovery from anaerobic digester supernatant at an enhanced biological phosphorus removal wastewater treatment plant. *J. Environ. Eng. Sci* **4**: 265–77.

Brooks, J.P., Maxwell, S.L., Rensing, C., Gerba, C.P., Pepper, I.L. (2007) Occurrence of antibiotic resistant bacteria and endotoxin associated with the land application of biosolids. *Canadian Journal of Microbiology* **53**(5): 616-22.

Broszat, M., Nacke, H., Blasi, R., Siebe, C., Huebner, J., Daniel, R., Grohmann, E. (2014) Wastewater irrigation increases the abundance of potentially harmful Gammaproteobacteria in soils in Mezquital valley, Mexico. *Appl Environ Microbiol* **80**(17): 5282–91.

Brown, K.D., Kulis, J., Thomson, B., Chapman, T.H., Mawhinney, D.B. (2006) Occurrence of antibiotics in hospital, residential, and dairy effluent, municipal wastewater, and the Rio Grande in New Mexico. *Sci Total Environ* **366**: 772–83.

Burch, T.R., Sadowsky, M.J., LaPara, T.M. (2013) Aerobic digestion reduces the quantity of antibiotic resistance genes in residual municipal wastewater solids. *Front Microbiol* **4**(17): 1–9.

Burow, L.C., Mabbett, A.N., McEwan, A.G., Bond, P.L., Blackall, L.L. (2008) Bioenergetic models for acetate and phosphate transport in bacteria important in enhanced biological phosphorus removal. *Environ. Microbiol* **10**: 87–98.

Chen, M., Wang, W., Feng, Y., Zhu, X., Zhou, H., Tan, Z., Li, X. (2014) Impact resistance of different factors on ammonia removal by heterotrophic nitrification-aerobic denitrification bacterium *Aeromonas sp.* HN-02. *Bioresour Technol* **167**: 456-61.

Cheng, H.-Y., Chen, Y.-F., Peng, H.-L. (2010) Molecular characterization of the PhoPQ-PmrD-PmrAB mediated pathway regulating polymyxin B resistance in *Klebsiella pneumoniae* CG43. *Journal of Biomedical Science 17*(1): 60. http://doi.org/10.1186/1423-0127-17-60.

Chonova, T., Keck, F., Labanowski, J., Montuelle, B., Rimet, F., Bouchez, A. (2016) Separate treatment of hospital and urban wastewaters: A real scale comparison of effluents and their effect on microbial communities. *Sci Total Environ* **542**(Pt A): 965–75.

Chopra, I., & Roberts, M. (2001) Tetracycline antibiotics: Mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiology and Molecular Biology Reviews* **65**(2): 232–60.

Ćirić, S., Petrović, O., Milenković, D. (2010) Low-nutrient R2A medium in monitoring microbiological quality of drinking water. *Chemical Industry & Chemical Engineering Quarterly* **16**(1): 39−45.

Claesson, M.J., Wang, Q., O'Sullivan, O., Greene-Diniz, R., Cole, J.R., Ross, R.P., O'Toole, P.W. (2010) Comparison of two next-generation sequencing technologies for resolving highly complex microbiota composition using tandem variable 16S rRNA gene regions. *Nucleic Acids Research* ***38***(22), e200.

Clara, M., Kreuzinger, N., Strenn, B., Gans, O., Kroiss, H. (2005) The solids retention time- a suitable design parameter to evaluate the capacity of wastewater treatment plants to remove micropollutants. *Water Research* **39**(1): 97-106.

Clinical and Laboratory Standards Institute, (2011) M100–S16. Performance standards for antimicrobial susceptibility testing, sixteenth informational supplement. *Wayne PA: CLSI.*

Comeau, Y., Hall, K.J., Hancock, R.E.W., Oldham, W.K. (1986) Biochemical model for enhanced biological phosphorus removal. *Water Research* **20**: 1511–21.

Cook, V.J., Turenne, C.Y., Wolfe, J., Pauls, R., Kabani, A. (2003) Conventional methods versus 16S ribosomal DNA sequencing for identification of Nontuberculous *Mycobacteria*: Cost analysis. *Journal of Clinical Microbiology* ***41***(3): 1010–5.

Cosenza A., Di Bella G., Mannina G., Torregrossa M. (2013) The role of EPS in fouling and foaming phenomena for a membrane bioreactor. *Bioresour. Technol* **147**: 184–92.

Cydzik-Kwiatkowska, A. & Zielińska, M. (2016) Bacterial communities in full-scale wastewater treatment systems. *World J Microbiol Biotechnol* **32**(4): 66.

Davies, J. & Davies, D. (2010) Origins and evolution of antibiotic resistance. *Microbiol Mol Biol Rev* **74**(3): 417–33.

Decrey, L., Udert, K.M., Tilley, E., Pecson, B.M., Kohn, T. (2011) Fate of the pathogen indicators phage ΦX174 and Ascaris suum eggs during the production of struvite fertilizer from source separated urine. *Water Research* **45**(16): 4960-72.

De Sotto, R.B., Medriano, C.A.D., Salcedo, D.E., Lee, H., Cho, Y., Kim, S. (2016) Effects of solids retention time on the fate of tetracycline resistance in SBRs for the treatment of carcass leachate. *Journal of Environmental Management* **181**: 298-303.

Devarajan, N., Laffite, A., Mulaji, C. K., Otamonga, J.-P., Mpiana, P. T., Mubedi, J. I., … Poté, J. (2016) Occurrence of Antibiotic Resistance Genes and Bacterial Markers in a Tropical River Receiving Hospital and Urban Wastewaters. *PLoS ONE 11*(2), e0149211.

Di Bella G., Torregrossa M., Viviani G. (2011) The role of EPS concentration in MBR foaming: Analysis of a submerged pilot plant. *Bioresour. Technol* **102**: 1628–35.

Donato, J.J., Moe, L.A., Converse, B.J., Smart, K.D., Berklein, F.C., McManus, P.S., Handelsman, J. (2010) Metagenomic analysis of apple orchard soil reveals antibiotic resistance genes encoding predicted bifunctional proteins. *Appl Environ Microbiol* **76**(13): 4396–4401.

Duong, H.A., Pham, N.H., Nguyen, H.T., Hoang, T.T., Pham, H.V., Pham, V.C., Berg, M., Giger, W., Alder, A.C. (2008) Occurrence, fate and antibiotic resistance of fluoroquinolone antibacterials in hospital wastewaters in Hanoi, Vietnam. *Chemosphere* **72**(6): 968–73.

Eaton, A.D., Clesceri, L.S., Rice, E.W., Greenberg, A.E. (2005) Standard methods for the examination of water and wastewater. 21st edition. American Public Health Association, American Water Works Association, Water Environment Federation.

Eliopoulos, G.M. & Huovinen, P. (2001) Resistance to trimethoprim-sulfamethoxazole. *Clinical infectious Diseases* **32**(11): 1608-14.

European Committee on Antimicrobial Susceptibility Testing (EUCAST) Breakpoint tables for interpretation of MICs and zone diameters. Version 1.0.

Fàbrega, A., Madurga, S., Giralt, E., Vila, J. (2009) Mechanism of action and resistance to quinolones. *Microbial Biotechnology* **2**(1): 41-60.

Fars, S., Oufdou, K., Nejmeddine, A., Hassani, L., Melloul, A.A., Bousselhaj, K., et al. (2005) Antibiotic resistance and survival of faecal coliforms in activated sludge system in a semi-arid region (Beni Bellal, Morocco). *World Journal of Microbiology and Biotechnology* **4**: 493-500.

Fenu, A., Guglielmi, G., Jimenez, J., Spérandio, M., Saroj, D., Lesjean, B., Brepols, C., Thoeye, C., Nopens, I. (2010) Activated sludge model (ASM) based modeling of membrane bioreactor (MBR) processes: A critical review with special regard to MMR specificities. *Water Research* **44**: 4272−94.

Ferguson, R.W. & Weissfeld, A.S. (1984) Comparison of the suitability of three common bacterial media for susceptibility testing of trimethoprim-sulfamethoxazole. *Journal of Clinical Microbiology*

19: 85-6.

Fernandes, R., Amador, P., Prudencio, C. (2013) β-Lactams: chemical structure, mode of action and mechanisms of resistance. *Reviews in Medical Microbiology* **24**(1): 7-17.

Ferreira da Silva, M., Tiago, I., Verissimo, A., Boaventura, R.A.R., Nunes, O.C., Manaia, C.M. (2006) Antibiotic resistance of enterococci and related bacteria in an urban wastewater treatment plant. *FEMS Microbiol Ecol* **55**: 322–9.

Figueira, V., Serra, E., Manaia, C.M. (2011) Differential patterns of antimicrobial resistance in population subsets of Escherichia coli isolated from waste- and surface waters. *The Science of the Total Environment* **409**(6): 1017-23.

Galand, P.E., Casamayor, E.O., Kirchman, D.L., Lovejoy, C. (2009) Ecology of the rare microbial biosphere of the Arctic Ocean. *PNAS* **106**: 22427–32.

Gallert, C., Fund, K. Winter, J. (2005) Antibiotic resistance of bacteria in raw and biologically treated sewage and in groundwater below leaking sewers. *Appl Microbiol Biotechnol* **69**: 106–112.

Galvin, S., Boyle, F., Hickey, P., Vellinga, A., Morris, D., Cormican, M. (2010) Enumeration and characterization of antimicrobial resistant Escherichia coli bacteria in effluent from municipal, hospital, and secondary treatment facility sources. *Appl Environ Microbiol* **76**(14): 4772–9.

Gao, P., Munir, M., Xagoraraki, I. (2012) Correlation of tetracycline and sulfonamide antibiotics with corresponding resistance genes and resistant bacteria in a conventional municipal wastewater treatment plant. *Sci Total Environ* **421-422**: 173–83.

Garcia-Armisen, T., Vercammen, K., Passerat, J., Triest, D., Servais, P., Cornelis, P. (2011) Antimicrobial resistance of heterotrophic bacteria in Sewage-contaminated rivers. *Water Research* **45**: 788-96.

Gatica, J. & Cytryn, E. (2013) Impact of treated wastewater irrigation on antibiotic resistance in the soil microbiome. *Environ Sci Pollut Res Int* **20**(6): 3529–38.

Gensberger, E.T., Gössl, E.M., Antonielli, L., Sessitsch, A., Kostić, T. (2015) Effect of different heterotrophic plate count methods on the estimation of the composition of the culturable microbial community. *PeerJ* **3**: e862.

Gibreel, A., & Sköld, O. (1999) Sulfonamide resistance in clinical isolates of *Campylobacter*

jejuni: Mutational changes in the chromosomal dihydropteroate synthase. *Antimicrobial Agents and Chemotherapy* ***43***(9): 2156–60.

Gobel, A., Thomsen, A., McArdell, C.S., Joss, A., Giger, W. (2005) Occurrence and sorption behavior of sulfonamides, macrolides, and trimethoprim in activated sludge treatment. *Environ Sci Technol* **39**: 3981–9.

Goñi-Urriza, M., Capdepuy, M., Arpin, C., Raymond, N., Caumette, P., Quentin, C. (2000) Impact of an urban effluent on antibiotic resistance of riverine *Enterobacteriaceae* and *Aeromonas spp*. *Appl Environ Microbiol* **66**(1): 125–32.

Gonzalez, L.S. & Spencer, J.P. (1998) Aminoglycosides: A practical review. *Am Fam Physician* **58**(8): 1811-20.

Grape, M., Motakefi, A., Pavuluri, S., Kahlmeter, G. (2007) Standard and real-time multiplex PCR methods for detection of trimethoprim resistance *dfr* genes in large collections of bacteria**.** *Clinical Microbiology and Infection* **13**(11): 1112–8.

Guardabassi, L., Danilo, M.A., Wong, L.F., Dalsgaard, A. (2002) The effects of tertiary wastewater treatment on the prevalence of antimicrobial resistant bacteria. *Water Research* **36**: 1955–64.

Guo, H., Wyart, Y., Perot, J., Nauleau, F., Moulin, P. (2010) Low-pressure membrane integrity tests for drinking water treatment: A review. Water Res **44**(1): 41-57.

Hai, F.I., Riley, T., Shawkat, S., Magram, S.F., Yamamoto, K. (2014) Removal of pathogens by membrane bioreactors: A review of the mechanisms, influencing factors and reduction in chemical disinfectant dosing. *Water* ***6***: 3603-30.

Hall, E.R., Monti, A., Mohn, W.W. (2010) A comparison of bacterial populations in enhanced biological phosphorus removal processes using membrane filtration or gravity sedimentation for solids–liquid separation. *Water Research* **44**: 2703-14.

Hall, E.R., Monti, A., Mohn, W.W. (2011) Production and characterization of foam in the anoxic zone of a membrane-enhanced biological phosphorus removal process. *Water Environment Research* **83**(2): 173-83.

Hamilton, L. & Larsen, H. (2010) Clinical microbiology. In Hubbard, J.D. (Editor) A concise review of clinical laboratory science. Lippincott Williams & Wilkins. Second edition.

Han, N., Sheng, D., Xu, H. (2012) Role of *Escherichia coli* strain subgroups, integrons, and integron-associated gene cassettes in dissemination of antimicrobial resistance in aquatic environments of Jinan, China. *Water Science and Technology* **66**(11): 2385-92.

Harb, M. & Hong, P-Y. (2017) Molecular-based detection of potentially pathogenic bacteria in membrane bioreactor (MBR) systems treating municipal wastewater: A case study. *Environ Sci Pollut Res* **24**: 5370–80.

Harris, S., Morris, C., Morris, D., Cormican, M., Cummins, E. (2013) The effect of hospital effluent on antimicrobial resistant *E. coli* within a municipal wastewater system. *Environ Sci Process Impacts* **15**(3): 617–22.

Harwood, V.J., Brownwell, M., Perusek, W., Whitlock, J.E. (2001) Vancomycin-resistant Enterococcus spp. isolated from wastewater and chicken feces in the United States. *Appl Environ Microbiol* **67**: 4930–33.

Hatosy, S.M. & Martiny, A.C. (2015) The ocean as a global reservoir of antibiotic resistance genes. *Appl Environ Microbiol* **81**(21): 7593–9.

He, S., Gall, D.L., McMahon, K.D. (2007) "*Candidatus Accumulibacter*" population structure in enhanced biological phosphorus removal sludges as revealed by polyphosphate kinase genes. *Appl Environ Microbiol* **73**: 5865–74.

Herzog, B., Lemmer, H., Horn, H., Müller, E. (2013) Characterization of pure cultures isolated from sulfamethoxazole-acclimated activated sludge with respect to taxonomic identification and sulfamethoxazole biodegradation potential. *BMC Microbiology* **13**, 276.

Heylen, K., Lebbe, L., De Vos, P. (2008) *Acidovorax caeni sp. nov.,* a denitrifying species with genetically diverse isolates from activated sludge. *Int J Syst Evol Microbiol* **58**(Pt 1): 73-7.

Hollenbeck, B.L., & Rice, L.B. (2012) Intrinsic and acquired resistance mechanisms in *Enterococcus*. *Virulence* 3(5): 421–33.

Holzel, C.S., Schwaiger, K., Harms, K., Kuchenhoff, H., Kunz, A., Meyer, K., et al. (2010) Sewage sludge and liquid pig manure as possible sources of antibiotic resistant bacteria. *Environmental Research* **110**(4): 318-26.

Hu, Q., Zhang, X.X., Jia, S., Huang, K., Tang, J., Shi, P., Ye, L., Ren, H. (2016) Metagenomic insights into ultraviolet disinfection effects on antibiotic resistome in biologically treated

wastewater. *Water Research* **101**: 309–17.

Huang, H.K & Tseng, S.K. (2001) Nitrate reduction by Citrobacter diversus under aerobic environment. *Appl Microbiol Biotechnol* **55**(1): 90-4.

Huang, J., Hu, H., Tang, F., Li, Y., Lu, S., Lu, Y. (2011) Inactivation and reactivation of antibiotic resistant bacteria by chlorination in secondary effluents of a municipal wastewater treatment plant. *Water Research* **45**: 2775-81.

Huang, J.J., Hu, H.Y., Lu, S.Q., Li, Y., Tang, F., Lu, Y., Wei, B. (2012) Monitoring and evaluation of antibiotic-resistant bacteria at a municipal wastewater treatment plant in China. *Environment International* **42**(1): 31-6.

Huovinen, P., Sundström, L., Swedberg, G., Sköld, O. (1995) Trimethoprim and sulfonamide resistance. *Antimicrobial Agents and Chemotherapy* ***39***(2): 279–89.

Iorhemen, O.T., Hamza, R.A., Tay, J.H. (2016) Membrane bioreactor (MBR) technology for wastewater treatment and reclamation: Membrane fouling. *Membranes* ***6***, 33.

Iversen, A., Kuhn, I., Franklin, A., Mollby, R. (2002) High prevalence of vancomycin-resistant Enterococci in swedish sewage. *Applied and Environmental Microbiology* **68**(6): 2838-42.

Iwane, T., Urase, T., Yamamoto, K. (2001) Possible impact of treated wastewater discharge on incidence of antibiotic resistant bacteria in river water. *Water Sci Technol* **43**(2): 91–9.

Jacangelo, J.G., Laine, J-M., Carns, K.E., Cummings, E.W., Mallevialle, J. (1991) Low-pressure membrane filtration for removing Giardia and microbial indicators. *AWWA* **83**(9): 97-106.

Jackson, R.W., Vinatzer, B., Arnold, D.L., Dorus, S., Murillo, J. (2011) The influence of the accessory genome on bacterial pathogen evolution. *Mob Genet Elements* **1**(1): 55–65.

Jia, A., Wan, Y., Xiao, Y., Hu, J. (2012) Occurrence and fate of quinolone and fluoroquinolone antibiotics in a municipal sewage treatment plant. *Water Research* **46**(2): 387-94.

Jung, C.M., Heinze, T.M., Strakosha, R., Elkins, C.A., Sutherland, J.B. (2009) Acetylation of fluoroquinolone antimicrobial agents by an *Escherichia coli* strain isolated from a municipal wastewater treatment plant. *Journal of Applied Microbiology* **106**: 564–71.

Jury, K.L., Khan, S.J., Vancov, T., Stuetz, R.M., Ashbolt, N.J. (2011) Are sewage treatment plants promoting antibiotic resistance? *Environmental Science and Technology* **41**(3): 243-70.

Kim, S., Eichhorn, P., Jensen, J.N., Weber, A.S., Aga, D.S. (2005) Removal of antibiotics in wastewater: Effect of hydraulic and solid retention times on the fate of tetracycline in the activated sludge process. *Environ Sci Technol* **39**: 5816–23.

Kim, J., Lim, J., Lee, C. (2013) Quantitative real-time PCR approaches for microbial community studies in wastewater treatment systems: Applications and considerations. *Biotech Advances* **31**(8): 1358–73.

Kim, S. & Aga, D.S. (2007) Potential ecological and human health impacts of antibiotics and antibiotic resistant bacteria from wastewater treatment plants. *J Toxicol Environ Health* Part B. **10**: 559–73.

Kim, S., Jensen, J.N., Aga, D.S., Weber, A.S. (2007a) Fate of tetracycline resistant bacteria as a function of activated sludge process organic loading and growth rate. *Water Sci Technol* **55**(1-2): 291-7.

Kim, S., Aga, D.S., Jensen, J.N., Weber, A.S. (2007b) Effect of sequencing batch reactor operation on presence and concentration of tetracycline-resistant organisms. Water Environment Research: a Research Publication of the Water Environment Federation **79**: 2287-97.

Kim, Y.M., Cho, H.U., Lee, D.S., Park, D., Park, J.M. (2011) Influence of operational parameters on nitrogen removal efficiency and microbial communities in a full-scale activated sludge process. *Water Research* **45**(17): 5785-95.

Kim, S., Park, H., Chandran, K. (2010) Propensity of activated sludge to amplify or attenuate tetracycline resistance genes and tetracycline resistant bacteria: A mathematical modeling approach. *Chemosphere* **78**: 1071–7.

Koczura, R., Mokracka, J., Jabłońska, L., Gozdecka, E., Kubek, M., Kaznowski, A. (2012) Antimicrobial resistance of integron-harboring *Escherichia coli* isolates from clinical samples, wastewater treatment plant and river water. *Sci Total Environ* **414**: 680–5.

Kong, Y., Xia, Y., Nielsen, J.L., Nielsen, P.H. (2007) Structure and function of the microbial community in a full-scale enhanced biological phosphorus removal plant. *Microbiology **153***(12): 4061-73.

Kotlarska, E., Łuczkiewicz, A., Pisowacka, M., Burzyński, A. (2015) Antibiotic resistance and prevalence of class 1 and 2 integrons in Escherichia coli isolated from two wastewater treatment plants, and their receiving waters (Gulf of Gdansk, Baltic Sea, Poland). *Environ Sci Pollut Res Int*

22: 2018–30.

Kovalova, L., Siegrist, H., Gunten, U.V., Eugster, J., Hagenbuch, M., Wittmer, A., Moser, R., McArdell, C.S. (2013) Elimination of micropollutants during post-treatment of hospital wastewater with powdered activated carbon, ozone, and UV. *Environ Sci Technol* **47**(14): 7899–7908.

Krishnaswamy, U., Muthuchamy, M., Perumalsamy, L. (2011) Biological removal of phosphate from synthetic wastewater using bacterial consortium. *Iranian Journal of Biotechnology* **9**(1): 37-49.

Kulaev, I.S., Vagabov, V.M., Kulakovskaya, T.V. (2004) The biochemistry of inorganic polyphosphates. 2nd edition. *Wiley, Chichester.*

Kümmerer, K. & Henninger, A. (2003) Promoting resistance by the emission of antibiotics from hospitals and households into effluent. *Clin Microbiol Infect* **9**: 1203–14.

Kundu, P., Pramanik, A., Dasgupta, A., Mukherjee, S., Mukherjee, J. (2014) Simultaneous heterotrophic nitrification and aerobic denitrification by *Chryseobacterium sp.* R31 isolated from Abattoir wastewater. *BioMed Research International.* Article ID 436056, doi:10.1155/2014/436056.

Lachmayr, K.L., Kerkhof, L.J., DiRienzo, A.G., Cavanaugh, C.M., Ford, T.E. (2009) Quantifying nonspecific TEM β-Lactamase (bla_{TEM}) genes in a wastewater stream. *Applied and Environmental Microbiology* **75**(1): 203–11.

Lam, K.N., Cheng, J., Engel, K., Neufeld, J.D., Charles, T.C. (2015) Current and future resources for functional metagenomics. *Front Microbiol* **6**, 1196.

LaPara, T.M., Burch, T.R., McNamara, P.J., Tan, D.T., Yan, M., Eichmiller, J.J. (2011) Tertiary treated municipal wastewater is a significant point-source of antibiotic resistance genes into Duluth-Superior Harbor. *Environ Sci Technol* **45**: 9543–9.

Lawson C.E. (2014) Population dynamics and metabolic potential of a pilot-scale microbial community performing enhanced biological phosphorus removal. Masters Thesis. Department of Civil Engineering, University of British Columbia, Vancouver, Canada.

Lee, H., Yong, D., et al. (2006) Dissemination of 16S rRNA methylase-mediated highly amikacin-resistant isolates of *Klebsiella pneumoniae* and *Acinetobacter baumannii* in Korea. *Diagnostic Microbiology and Infectious Disease*. **56**(3):305-12.

Lee, S., & Hallam, S.J. (2009) Extraction of high molecular weight genomic DNA from soils and sediments. *Journal of Visualized Experiments.* JoVE no. 33. doi:10.3791/1569.

Lefkowitz, J.R. & Duran, M. (2009) Changes in antibiotic resistance patterns of *Escherichia coli* during domestic wastewater treatment. *Water Environ Res* **81**(9): 878–85.

Levin, G.V. & Shapiro, J. (1965) Metabolic uptake of phosphorus by wastewater organisms. *J. Water Pollut. Cont. Fed* **37**: 800-21.

Levy, S.B. (1992) Active efflux mechanisms for antimicrobial resistance. *Antimicrobial Agents and Chemotherapy* **36**(4): 695–703.

Li, B., Zhang, T., Xu, Z., Fang, H.H.P. (2009) Rapid analysis of 21 antibiotics of multiple classes in municipal wastewater using ultra performance liquid chromatography-tandem mass spectrometry. *Analytica Chimica Acta* **645**(1–2): 64-72.

Li, B. & Zhang, T. (2010) Biodegradation and adsorption of antibiotics in the activated sludge process. *Environ Sci Technol* **44**: 3468–73.

Li, D., Yu, T., Zhang, Y., Yang, M., Li, Z., Liu, M., Qi, R. (2010) Antibiotic resistance characteristics of environmental bacteria from an oxytetracycline production wastewater treatment plant and the receiving river. *Appl Environ Microbiol* **76**: 3444–51.

Li, B. & Wu, G. (2014) Effects of sludge retention times on nutrient removal and nitrous oxide emission in biological nutrient removal processes. *Int J Environ Res Public Health* **11**: 3553–69.

Li, A.D., Li, L.G., Zhang, T. (2015) Exploring antibiotic resistance genes and metal resistance genes in plasmid metagenomes from wastewater treatment plants. *Front Microbiol* **6**, 1025.

Lin, J., Nishino, K., Roberts M.C., Tolmasky, M., Aminov, R.I., Zhang, L. (2015) Mechanisms of antibiotic resistance. *Frontiers in Microbiology* **6**(34): 1-3.

Liu, G. & Wang, J. (2014) Role of solids retention time on complete nitrification: Mechanistic understanding and modeling. *J Environ Eng* **140** (1): 48–56.

Liu, M., Gill, J.J., Young, R., Elizabeth, J., Summer, E.J. (2015) Bacteriophages of wastewater foaming-associated filamentous Gordonia reduce host levels in raw activated sludge. *Sci Rep* **5**, 13754.

Lousada-Ferreira, M., van Lier, J.B., van der Graaf, J.H.J.M. (2016) Particle counting as surrogate measurement of membrane integrity loss and assessment tool for particle growth and

regrowth in the permeate of membrane bioreactors. Separation and Purification Technology **161**: 16–24.

Lu, Q. & Stoffella, P.S. (2012) Land application of biosolids in the USA: A review. *Appl Environ Soil Sci* Article ID: 201462.

Luczkiewicz, A., Jankowska, K., Fudala-Ksiazek, S., Olanczuk-Neyman, K. (2010) Antimicrobial resistance of fecal indicators in municipal wastewater treatment plant. *Water Research* **44**(17): 5089–97.

Lüddeke, F., He, S., Gallert, C., Winter, J., Gude, H., Loffler, H. (2015) Removal of total and antibiotic resistant bacteria in advanced wastewater treatment by ozonation in combination with different filtering techniques. *Water Research* **69**: 243–51.

Luo, Y., Guo, W., Ngo, H.H., Nghiem, L.D., Hai, F.I, Zhang, J., Liang, S., Xiaochang, C., Wang, X.C. (2014) A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. *Sci Total Environ* **473–474**(1): 619–41.

Ma, L., Zhang, X.X., Cheng, S., Zhang, Z., Shi, P., Liu, B., Wu, B., Zhang, Y. (2011) Occurrence, abundance and elimination of class 1 integrons in one municipal sewage treatment plant. *Ecotoxicol* **20**(5): 968–73.

Ma, Y., Metch, J.W., Yang, Y., Pruden, A., Zhang, T. (2016) Shift in antibiotic resistance gene profiles associated with nanosilver during wastewater treatment. *FEMS Microbiol Ecol* **92**(3): 1-8.

Ma, L., Zhang, X.X., Zhao, F., Wu, B., Cheng, S., Yang, L. (2013) Sewage treatment plant serves as a hot-spot reservoir of integrons and gene cassettes. *J Environ Biol* **34**: 391–9.

MacConkey, A.T. (1908) Bile salt media and their advantages in some bacteriological examinations. *J Hyg (Lond)* **8**(3): 322–34.

Mahmod, F. (2014) Novel methods to study intestinal microbiota. Masters Thesis. Norwegian University of Life Sciences, Department of Chemistry, Biotechnology and Food Science.

Malek, M.M., Amer, F.A., Allam, A.A., El-Sokkary, R.H., Gheith, T., Arafa, M.A. (2015) Occurrence of classes I and II integrons in *Enterobacteriaceae* collected from Zagazig University hospitals, Egypt. *Front. Microbiol* **6**, 601.

Mulvey, M., Mataseje, L.F. et al. (2016) Dissemination of *mcr*-1 colistin resistance gene. *The Lancet Infectious Diseases* **16**(3): 289-90.

Manaia, C.M., Novo, A., Coelho, B., Nunes, O.C. (2010) Ciprofloxacin resistance in domestic wastewater treatment plants. *Water, Air, and Soil Pollution* **208**(1-4): 335-43.

Marathe, N.P., Regina, V.R., Walujkar, S.A., Charna, S.S., Moore, E.R.B., Larsson, D.G.J., Shouche, Y.S. (2013) A treatment plant receiving waste water from multiple bulk drug manufacturers is a reservoir for highly multi-drug resistant integron-bearing bacteria. *PLoS One* **8**(10): e77310.

Martín, H.G., Ivanova, N., Kunin, V., Warnecke, F., Barry, K.W., McHardy, A.C., et al. (2006) Metagenomic analysis of two enhanced biological phosphorus removal (EBPR) sludge communities. *Nat Biotech* ***24***(10): 1263-9.

Martins da Costa, P., Vaz-Pires, P., Bernardo, F. (2006) Antimicrobial resistance in *Enterococcus spp.* isolated in inflow, effluent and sludge from municipal sewage water treatment plants. *Water Research* **40**(8): 1735–40.

Mathur, A., Kumari, J., Parashar, A.T.L., Chandrasekaran, N., Mukherjee, A. (2015) Decreased phototoxic effects of TiO_2 nanoparticles in consortium of bacterial isolates from domestic waste water. *PLoS One* ***10***(10): e0141301.

Matuschek, E., Brown, D.F.J., Kahlmeter, G. (2014) Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories. *Clinical Microbiology and Infection* **20**(4): O255-66.

Mazzucotelli, C.A., Durruty, I., Kotlar, C.E., Moreira, M.R., Ponce, A.G., Roura, S.I. (2014) Development of a microbial consortium for dairy wastewater treatment. *Biotechnology and Bioprocess Engineering* **19**: 221-30.

McGee, D.J., Coker, C., Harro, J.M., Mobley, H.L.T. (2001) Bacterial genetic exchange. *eLS*. John Wiley and Sons, Ltd.

McLain, J. E., Cytryn, E., Durso, L. M., Young, S. (2016) Culture-based methods for detection of antibiotic resistance in agroecosystems: Advantages, challenges, and gaps in knowledge. *J. Environ Qual* ***45***(2): 432-40.

Mellon, M., Benbrook. C., Benbrook, K.L. (2001) Hogging it: estimates of antimicrobial abuse in livestock. Cambridge, MA: *Union of Concerned Scientists Publications*. Available at http://www.ucsusa.org/assets/documents/food_and_agriculture/hog_front148.pdf.

McNeil, J. C., Hulten, K. G., Kaplan, S. L., & Mason, E. O. (2011) Mupirocin resistance in *Staphylococcus aureus* causing recurrent skin and soft tissue infections in children. *Antimicrobial Agents and Chemotherapy* ***55***(5): 2431–2433.

Mehnert-Kay, S.A. (2005) Diagnosis and management of uncomplicated urinary tract infections. *Am Fam Physician* **72**(3): 451-6.

Metcalf, L. & Eddy H.P. (revised by Tchobanoglous, G., Burton, F.L., Stensel, H.D.) (2003) Wastewater engineering, treatment and reuse. 4th edition, McGraw-Hill, New York.

Mezrioui, N., & Baleux, B. (1994) Resistance patterns of *E. coli* strains isolated from domestic sewage before and after treatment in both aerobic lagoon and activated sludge. *Water Research* **28**: 2399–2406.

Michael, I., Rizzo, L., McArdell, C.S., Manaia, C.M., Merlin, C., Schwartz, T., et al. (2013) Urban wastewater treatment plants as hotspots for the release of antibiotics in the environment: A review. *Water Research* **47**(3): 957-95.

Miller, J.H., Noval, J.T., Knocke, W.R., Pruden, A. (2014) Elevation of antibiotic resistance genes at cold temperatures: Implications for winter storage of sludge and biosolids. *Lett Appl Microbiol* **59**(6): 587–93.

Minh, T.B., Leung, H.W., Loi, I.H., Chan, W.H., So, M.K., Mao, J.Q., et al. (2009) Antibiotics in the Hong Kong metropolitan area: Ubiquitous distribution and fate in Victoria Harbour. *Marine Pollution Bulletin* **58**(7): 1052-62.

Mino, T., van Loosdrecht, M.C.M., and Heijnen, J.J. (1998) Microbiology and biochemistry of the enhanced biological phosphorus removal process. *Water Research* **32**(11): 3193–3207.

Mokracka, J., Koczura, R., Kaznowski, A. (2012) Multiresistant *Enterobacteriaceae* with class 1 and class 2 integrons in a municipal wastewater treatment plant. *Water Research* **46**(10): 3353-63.

Monti, A. (2006) Impact and potential of membrane solids-liquid separation in biological nutrient removal processes. Ph.D. Dissertation. Department of Civil Engineering, University of British Columbia, Vancouver, Canada.

Monti, A., Hall, E.R., Koch, F.A., Dawson, R.N., Husain, H., Kelly, H.G. (2007) Toward a high-rate enhanced biological phosphorus removal process in a membrane-assisted bioreactor. *Water*

Environment Research **79**(6): 675-86.

Morar, M., & Wright, G.D. (2010) The genomic enzymology of antibiotic resistance. *Annual Review of Genetics* **44**(1): 25-51.

Moretti, C.J., Das, D., Kistner, B.T., Gullicks, H., Tse Hung, Y-T. (2011) Activated sludge and other aerobic suspended culture processes. *Water* **3**: 806-18.

Moura, A., Pereira, C., Henriques, I., Correia, A. (2012) Novel gene cassettes and integrons in antibiotic resistant bacteria isolated from urban wastewaters. *Research in Microbiology* **163**(2): 92-100.

Munck, C., Albertsen, M., Telke, A., Ellabaan, M., Nielsen, P.H., Sommer, M.O. (2015) Limited dissemination of the wastewater treatment plant core resistome. *Nature Communications* **6**, 8452.

Munita, J.M., & Arias, C.A. (2016) Mechanisms of antibiotic resistance. *Microbiology Spectrum* **4**(2), doi: 10.1128/microbiolspec.VMBF–0016–2015.

Munir, M., & Xagoraraki, I. (2011) Levels of antibiotic resistance genes in manure, biosolids, and fertilized soil. *J Environ Qual* **40**(1): 248-55.

Munir, M., Wong, K., Xagoraraki, I. (2011) Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. *Water Research* **45**(2): 681–93.

Nagulapally, S.R., Ahmad, A., Henry, A., Marchin, G.L., Zurek, L., Bhandari, A. (2009) Occurrence of ciprofloxacin-, trimethoprim-sulfamethoxazole- and vancomycin-resistant bacteria in a municipal wastewater treatment plant. *Water Environ Res* **81**: 82–90.

Naili, O., Bedouins, M. Benmmar, L. (2015a) Screening of bacteria from activated sludge for phosphorus removal from wastewater. *J. Appl. Environ. Biol. Sci* **5**(5): 1-5.

Naili, O., Benounis, M., Darbouche, A. Kheddouma, A. (2015b) Biological denitrification using pure cultures isolated from wastewater treatment. *J. Biol. Environ.Sci* **9**(25): 33-9.

Nandi, S., Maurer, J.J., Hofacre, C., Summers, A.O. (2004) Gram- positive bacteria are a major reservoir of class 1 antibiotic resistance integrons in poultry litter. *Proc. Natl. Acad. Sci.* U.S.A. **101**(18): 7118–22.

Nakajima J. & Mishima I. (2005) Measurement of foam quality of activated sludge in MBR process. *Acta Hydrochim. Hydrobiol* **33**: 232–9.

Narayanasamy, S., Muller, E.E., Sheik, A.R, Wilmes, P. (2015) Integrated omics for the identification of key functionalities in biological wastewater treatment microbial communities. *Microb Biotechnol* **8**(3): 363–8.

Negreanu, Y., Pasternak, Z., Jurkevitch, E., Cytryn, E. (2012) Impact of treated wastewater irrigation on antibiotic resistance in agricultural soils. *Environ Sci Technol* **46**(9): 4800–8.

Nelson, M.C., Morrison, H.G., Benjamino, J., Grim, S. L., Graf, J. (2014) Analysis, optimization and verification of illumina-generated 16S rRNA gene amplicon surveys. *PLoS One* **9**(4), e94249.

Neyestani, M., Dickenson, E., McLain, J., Robleto, E., Rock, C., Gerrity, D. (2017). Impacts of solids retention time on trace organic compound attenuation and bacterial resistance to trimethoprim and sulfamethoxazole. *Chemosphere **182***: 149-58.

Ng, L.K., Mulvey, M.R., Martin, I., Peters, G.A., Johnson, W. (1999) Genetic characterization of antimicrobial resistance in Canadian isolates of Salmonella serovar Typhimurium DT104. *Antimicrob. Agents Chemother* **43**(12): 3018–21.

Nielsen, P.H., Mielczarek, A.T., Kragelund, C., Nielsen, J.L., Saunders, A.M., Kong, Y., et al. (2010) A conceptual ecosystem model of microbial communities in enhanced biological phosphorus removal plants. *Water Research* **44**: 5070–88.

Nogueira, R. & Melo, L.F. (2006) Competition between *Nitrospira spp*. and *Nitrobacter spp*. in nitrite-oxidizing bioreactors. *Biotechnol. Bioeng* **95**: 169–75.

Nouha, K., Kumar, R.S., Tyagi, R.D. (2016) Heavy metals removal from wastewater using extracellular polymeric substances produced by *Cloacibacterium normanense* in wastewater sludge supplemented with crude glycerol and study of extracellular polymeric substances extraction by different methods. *Bioresour Technol* **212**:120-9.

Noyes, N.R., Yang, X., Linke, L.M., Magnuson, R.M., Cook, S.R., Zaheer, R., Yang, H., Woerner, D.R., Geornaras, I., McArt, J.A., Gow, S.P., Ruiz, J., Jones, K.L., Boucher, C.A., McAllister, T.A., Belk, K.E., Morley, P.S. (2016) Characterization of the resistome in manure, soil and wastewater from dairy and beef production systems. *Sci Rep* **6**, 24645.

Oehmen, A., Lemos, P.C., Carvalho, G., Yuan, Z., Keller, J., Blackall, L.L., et al. (2007) Advances in enhanced biological phosphorus removal: From micro to macro scale. *Water Research* **41**(11): 2271-2300.

Oh, H., Lee, J., Kim, K., Kim, J., Choung, Y., Park, J. (2009) A novel laboratory cultivation method to examine antibiotic-resistance-related microbial risks in urban water environments. *Water Sci Technol* **59**(2): 347-52.

Okoh, A.I. & Igbinosa, E.O. (2010) Antibiotic susceptibility profiles of some Vibrio strains isolated from wastewater final effluents in a rural community of the Eastern Cape province of South Africa. *BMC Microbiol* **10**, 143.

Olaniran, A.O., Nzimande, S.B., Mkize, N.G. (2015) Antimicrobial resistance and virulence signatures of *Listeria* and *Aeromonas* species recovered from treated wastewater effluent and receiving surface water in Durban, South Africa. *BMC Microbiol* **15**, 234.

Opazo, A., Dominguez, M., Bello, H., Amyes, S., Gonzalez-Rocha, G. (2012) OXA-type carbapenemases in *Acinetobacter baumannii* in South America. *J Infect Dev Ctries* **6**(4): 311-6.

Oved, T., Shaviv, A., Golderath, T., Mandelbaum, R.T., Minzi, D. (2001) Influence of effluent irrigation on community composition and function of ammonia-oxidizing bacteria in soil. *Appl Environ Microbiol* **67**(8): 3426–33.

Pal, P., Khairnar, K., Paunikar, W.N. (2014) Causes and remedies for filamentous foaming in activated sludge treatment. *Global NEST Int J* **16**(4): 762–72.

Parker, D., Geary, S., Jones, G., McIntyre, L., Oppenheim, S., Pedregon, V., Pope, R., Richards, T., Voigt, C., Volpe, G., Willis, J., Witzgall, R. (2003) Making classifying selectors work for foam control in the activated sludge process. *Water Environ Res* **75**(1): 83–91.

Parsley, L.C., Consuegra, E.J., Kakirde, K.S., Land, A.M., Harper, W.F., Liles, M.R. (2010) Identification of diverse antimicrobial resistance determinants carried on bacterial, plasmid, or viral metagenomes from an activated sludge microbial assemblage. *Appl Environ Microbiol* **76**(11): 3753–57.

Patureau, D., Bernet, N., Moletta, R. (1997) Combined nitrification and denitrification in a single aerated reactor using the aerobic denitrifier *Commonas sp.* strain SGLY2. *Water Research* **31**(6): 1363-70.

Peccia, J. & Westerhoff, P. (2015) We should expect more out of our sewage sludge. *Environ. Sci. Technol* ***49***(14): 8271–6.

Pei, R., Kim, S., Carlson, K.H., Pruden, A. (2006) Effect of river landscape on the sediment

concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). *Water Research* **40**(12): 2427-35.

Peng, X., Tan, J., Tang C., Yu, Y., Wang, Z. (2008) Multiresidue determination of fluoroquinolone, sulfonamide, trimethoprim and chloramphenicol antibiotics in urban waters in China. *Environmental Toxicology and Chemistry* **27**(1): 73–9.

Pereira, H., Lemos, P.C., Reis, M.A.M., Crespo, J.P.S., Carrondo, M.J.T., Santos, H. (1996) Model for carbon metabolism in biological phosphorus removal processes based on *in vivo*^{13} C-NMR labelling experiments. *Water Research* **30**: 2128-38.

Pérez, S., Eichhorn, P., Aga, D.S. (2005) Evaluating the biodegradability of sulfamethazine, sulfamethoxazole, sulfathiazole, and trimethoprim at different stages of sewage treatment. *Environmental Toxicology and Chemistry* **24**(6): 1361-7.

Podnecky, N.L., Rhodes, K.A. et al. (2017) Mechanisms of resistance to folate pathway inhibitors in *Burkholderia pseudomallei*: Deviation from Norm. MBio 8(5). pii: e01357-17. doi: 10.1128/mBio.01357-17.

Poirel, L., Naas, T., Nordmann, P. (2010) Diversity, epidemiology, and genetics of class D beta-lactamases. *Antimicrob. Agents Chemother* **54**: 24–38.

Pruden, A., Pei, R., Storteboom, H., Carlson, K.H. (2006) Antibiotic resistance genes as emerging contaminants: Studies in Northern Colorado. *Environ. Sci. Technol* **40**(23): 7445–50.

Ramsden, S.J., Ghosh, S., Bohl, L.J., LaPara, T.M. (2010) Phenotypic and genotypic analysis of bacteria isolated from three municipal wastewater treatment plants on tetracycline- amended and ciprofloxacin amended growth media. *J. Appl. Microbiology* **109**: 1609–18.

Rahube, T.O., Mart, R., Scott, A., Tien, Y-C., Murray, R., Sabourin, L., Zhang, Y., Duenk, P., Lapen, D.R., Topp, E. (2014) Impact of fertilizing with raw or anaerobically digested sewage sludge on the abundance of antibiotic resistant coliforms, antibiotic resistance genes, and pathogenic bacteria in soil and on vegetables at harvest. *Appl Environ Microbiol* **80**(22): 6898-6907.

Rahube, T.O. & Yost, C.K. (2010) Antibiotic resistance plasmids in wastewater treatment plants and their possible dissemination into the environment. *African Journal of Biotechnology* **9**(54): 9183-90.

Reinthaler, F.F., Posch, J., Feierl, G., Wüst, G., Haas, D., Ruckenbauer, G., Mascher, F., Marth, E. (2003) Antibiotic resistance of *E. coli* in sewage and sludge. *Water Research* **37**: 1685–90.

Reasoner, D.J. & Geldreich, E.E. (1985) A new medium for enumeration and subculture of bacteria from potable water. *Appl. Environ. Microbiol* ***49***(1): 1-7.

Renew, J.E., & Huang, C. (2004) Simultaneous determination of fluoroquinolone, sulfonamide, and trimethoprim antibiotics in wastewater using tandem solid phase extraction and liquid chromatography-electrospray mass spectrometry. *Journal of Chromatography A* **1042**(1-2): 113-21.

Rice, L.B. (2008) Federal funding for the study of antimicrobial resistance in nosocomial pathogens: No ESKAPE. *J Infect Dis* **197**: 1079-81.

Rijal, G.K., Zmuda, J.T., Gore, R., Abedin, Z., Granato, T., Kollias, L., Lanyon, R. (2009) Antibiotic resistant bacteria in wastewater processed by the Metropolitan water reclamation district of Greater Chicago system. *Water Science and Technology* **59**(12): 2297-2304.

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagote, C., Ploy, M.C., Michael, I., Fatta-Kassinos, D. (2013) Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. *Sci Total Environ* **447**: 345–60.

Robinson, S., Abdullah, S.Z., Bérubé, P., Le-Clech, P. (2016) Aging of membranes for water treatment: Linking changes to performance. *Journal of Membrane Science* **503**: 177-87.

Roume, H., Heintz-Buschart, A., Muller, E.E., May, P., Satagopam, V.P., Laczny, C.C., et al. (2015) Comparative integrated omics: Identification of key functionalities in microbial community-wide metabolic networks. *npj Biofilms and Microbiomes* **1**: 1–11.

Rieuwerts, J. (2015) The elements of environmental pollution. Routledge. Edition 1st.

Santoro, D.O., Cardoso, A.M., Coutinho, F.H., Pinto, L.H., Vieira, R.P., Albano, R.M., Clementino, M.M. (2015) Diversity and antibiotic resistance profiles of *Pseudomonads* from a hospital wastewater treatment plant. *J Appl Microbiol* **119**(6): 1527–40.

Schmieder, R. & Edwards, R. (2012) Insights into antibiotic resistance through metagenomic approaches. *Future Microbiol* **7**(1): 73–89.

Schwartz, T., Kohnen, W., Jansen, B., Obst, U. (2003) Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. *FEMS*

Microbiol Ecology **43**: 325–35.

Sentchilo, V., Mayer, A.P., Guy, L., Miyazaki, R., Green Tringe, S., Barry, K., et al. (2013) Community-wide plasmid gene mobilization and selection. *Isme j* **7**(6): 1173-86.

Seviour, R.J., Mino, T., Onuki, M. (2003) The microbiology of biological phosphorus removal in activated sludge systems. *FEMS Microbiol. Rev* **27**: 99 – 127.

Sidat, M., Bux, F., Kasan, H.C. (1999) Polyphosphate accumulation by bacteria isolated from activated sludge. *Water SA* **25**(2): 175-80.

Sigala, J. & Unc, A. (2012) A PCR-DGGE approach to evaluate the impact of wastewater source on the antibiotic resistance diversity in treated wastewater effluent. *Water Science and Technology* **65**(7): 1323-31.

Simo Tchunite, P.L., Stalder, T., Venditti, S., Ngandjio, A., Dagot, C., Ploy, M.C., Barred, O. (2016) Characterisation of class 3 integrons with oxacillinase gene cassettes in hospital sewage and sludge samples from France and Luxembourg. *Int J Antimicrob Agents* **48**(4): 431–4.

Slater, H., Gouin, T., Leigh, M. (2011) Assessing the potential for rhizoremediation of PCB contaminated soils in northern regions using native tree species. *Chemosphere* **84**(2): 199-206.

Srivastava, S., Singh, V., Kumar, V., Verma, P.C., Srivastava, R., Basu, V., Rawat, A.K. (2008) Identification of regulatory elements in 16S rRNA gene of Acinetobacter species isolated from water sample. *Bioinformation* **3**(4): 173–6.

Stalder, T., Barraud, O., Jove, T., Casellas, M., Gaschet, M., Dagot, C., et al. (2014) Quantitative and qualitative impact of hospital effluent on dissemination of the integron pool. *Isme j* **8**(4): 768-77.

Stefani, F.O, Bell, T.H, Marchand, C., de la Providencia, I.E., El Yassimi, A., St-Arnaud, M., Hijri, M. (2015) Culture-dependent and independent methods capture different microbial community fractions in hydrocarbon-contaminated soils. PloS One **10**(6): e0128272.

Stokes, H.W. & Hall, R.M. (1992) The integron In1 in plasmid R46 includes two copies of the oxa2 gene cassette. *Plasmid* **28**(3): 225–34.

Strachan, C.M. & Davies, J. (2016) The whys and wherefores antibiotic resistance. In Silver, L.L & Bush, K. (Editors) Additional perspectives on antibiotics and antibiotic resistance. Cold Spring Harbor Perspective in Medicine.

Strateva, T., & Yordanov, D. (2009) Pseudomonas aeruginosa – a phenomenon of bacterial resistance. *Journal of Medical Microbiology* **58**(9): 1133-48.

Suhartono, S. (2016) Dissemination and persistence of plasmid located, integrin associated antibiotic resistant genes in wastewater treatment plant effluent and stream water bacteria. Ph.D. Dissertation.Department of Crop, Soil and Environmental Sciences, University of Arkansas, USA.1538.

Szczepanowski, R., Linke, B., Krahn, I., Gartemann, K-H., Gützkow, T., Eichler, W., Pühler, A., Schlüter, A. (2009) Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. *Microbial* **155**(Part 7): 2306–19.

Szczepanowski, R., Krahn, I., Pühler, A., Schlüter, A. (2004a) Different molecular rearrangements in the integron of the IncP-1**β** resistance plasmid pB10 isolated from a wastewater treatment plant result in elevated beta-lactam resistance levels. *Archives of Microbiology* **182**(6): 429-35.

Szczepanowski, R., Krahn, I., Linke, B., Goesmann, A., Pühler, A., Schlüter, A. (2004b) Antibiotic multiresistance plasmid pRSB101 isolated from a wastewater treatment plant is related to plasmids residing in phytopathogenic bacteria and carries eight different resistance determinants including a multidrug transport system. *Microbiology* **150**(Part 11): 3613-30.

Tang, C.M., Huang, Q.X., Yu, Y.Y., Peng, X.Z. (2009) Multiresidue determination of sulfonamides, macrolides, trimethoprim, and chloramphenicol in sewage sludge and sediment using ultrasonic extraction coupled with solid phase extraction and liquid chromatography-tandem mass spectrometry. *Chinese Journal of Analytical Chemistry* **37**(8): 1119-24.

Tennstedt, T., Szczepanowski, R., Braun, S., Pühler, A., Schlüter, A. (2003) Occurrence of integron-associated resistance gene cassettes located on antibiotic resistance plasmids isolated from a wastewater treatment plant. *FEMS Microbiol Ecol* **45**: 239–52.

Tenover, F.C. (2006) Mechanisms of antimicrobial resistance in bacteria. *Am J Infect Control* **34**(5 Suppl 1): S3-10; discussion S64-73.

Thomsen, T.R., Kong, Y., Nielsen, P.H. (2007) Ecophysiology of abundant denitrifying bacteria in activated sludge. *FEMS Microbiol. Ecol* **60**: 370–82.

Torres-Cortés, G., Millán, V., Ramírez-Saad, H.C., Nisa-Martínez, R., Toro, N., Martínez-Abarca,

F. (2011) Characterization of novel antibiotic resistance genes identified by functional metagenomics on soil samples. *Environ Microbiol* **13**: 1101–14.

Usha, P.T.A., Jose, S., Nisha, A.R. (2010) Antimicrobial drug resistance- A global concern. *Veterinary World* **3**(3): 138-9.

Uyaguari, M.I., Fichot, E.B., Scott, G.I., Norman, R.S. (2011) Characterization and quantitation of a novel β-lactamase gene found in a wastewater treatment facility and the surrounding coastal ecosystem. *Appl Environ Microbiol* **77**(23): 8226–33.

Vela, A.L., Collins, M.D., Lawson, P.A., García, N., Domínguez, L., Fernández-Garayzábal, J.F. (2005) Uruburuella suis gen. nov., sp. nov., isolated from clinical specimens of pigs. *Int J Syst Evol Microbiol* **55**(Part 2): 643-7.

Vieira, F.C.S., & Nahas, E. (2005) Comparison of microbial numbers in soils by using various culture media and temperatures. *Microbiological Research* **160**(2): 197-202.

Volkmann, H., Schwartz, T., Bischoff, P., Kirchen, S., Obst, U. (2004) Detection of clinically relevant antibiotic-resistance genes in municipal wastewater using real-time PCR (taqman). *J Microbiol Methods* **56**(2): 277–86.

Wagner, M., Amann, R., Lemmer, H., and Schleifer, K.H. (1993). Probing activated- sludge with oligonucleotides specific for Proteobacteria—inadequacy of culture-dependent methods for describing microbial community structure. *Appl Environ Microb* **59**: 1520–5.

Walston, S.E. (2013) Does increasing solids retention time during the wastewater treatment process affect the persistence of antibiotic resistance genes? Masters Thesis. University of Arizona, Arizona, US.

Wang, Z., Zhang, X-X., Huang, K., Miao, Y., Shi, P., Liu, B., Long, Ch., Li, A. (2013) Metagenomic profiling of antibiotic resistance genes and mobile genetic elements in a tannery wastewater treatment plant. *PloS One* **8**(10): e76079.

Watkinson, A. J., Murby, E.J., Costanzo, S.D. (2007) Removal of antibiotics in conventional and advanced wastewater treatment: Implications for environmental discharge and wastewater recycling. *Water Research* **41**(18): 4164–76.

Wentzel, M.C., Lotter, L.H., Loewenthal, R.E., Marais, G. (1986) Metabolic behaviour of Acinetobacter spp. in enhanced biological phosphorus removal - a biochemical model. *Water SA*

12(4): 209-24.

West, B.M., Liggit, P., Clemans, D.L., Francoeur, S.N. (2011) Antibiotic resistance, gene transfer, and water quality patterns observed in waterways near Cafo farms and wastewater treatment facilities. *Water, Air, and Soil Pollution* **217**(1-4): 473-89.

Winkler, M-K.H., Kröber, E., Mohn, W.W., Koch, F., Frigon, D. (2016), Comparison of microbial populations and foaming dynamics in conventional versus membrane enhanced biological phosphorous removal systems. *Water and Environment Journal* **30**: 102–12.

Wong, M-T., Mino, T., Seviour, R.J., Liu, W.T. (2005) In situ identification and characterization of the microbial community structure of full-scale enhanced biological phosphorus removal plants in Japan. *Water Research* **39**(13): 2901-14.

Wright, J.J., Lee, S, Zaikova, E., Walsh D.A., Hallam, S.J. (2009) DNA extraction from 0.22 µM sterivex filters and cesium chloride density gradient centrifugation. *Journal of Visualized Experiments* **31**: e1352.

Xia, S., Jia, R., Feng, F., Xie, K., Li, H., Jing, D., Xu, X. (2012) Effect of solids retention time on antibiotics removal performance and microbial communities in an A/O-MBR process. *Bioresour Technol* **106**: 36-43.

Xia, C.W., Ma, Y.J., Zhang, F. et al. (2014) A novel approach for phosphorus recovery and no wasted sludge in enhanced biological phosphorus removal process with external COD addition. *Appl Biochem Biotechnol* **172**(2): 820-8.

Xu, W., Zhang, G., Li, X., Zou, S., Li, P., Hu, Z., et al. (2007a) Occurrence and elimination of antibiotics at four sewage treatment plants in the Pearl River Delta (PRD), South China. *Water Research* **41**: 4526–34.

Xu, H., Davies, J., Miao, V. (2007b) Molecular characterization of class 3 integrons from *Delftia spp*. *J. Bacteriol* **189**(17): 6276-83.

Xu, M., Guo, J., Cen, Y., Zhong, X., Cao, W., Sun, G. (2005) *Shewanella decolorationis sp. nov.*, a dye-decolorizing bacterium isolated from activated sludge of a waste-water treatment plant. *Int J Syst Evol Microbiol* **55**(Part 1): 363-8.

Yang, Y., Li, B., Ju, F., Zhang, T. (2013a) Exploring variation of antibiotic resistance genes in activated sludge over a four-year period through a metagenomic approach. *Environmental*

Science & Technology **47**(18): 10197-205.

Yang, D., Wang, J., Qiu, Z., Jin, M., Shen, Z., Chen, Z., et al. (2013b) Horizontal transfer of antibiotic resistance genes in a membrane bioreactor. *Journal of Biotechnology* **167**(4): 441-7.

Yang, Y., Zhang, T., Zhang, X-X., Ming, L., Zhang, D-W., Gao, D-W., Zhu, H-G., Huang, Q-G., Fang, H.P. (2012) Quantification and characterization of β-lactam resistance genes in 15 sewage treatment plants from East Asia and North America. *Appl Microbiol Biotechnol* **95**(5): 1351-8.

Yang, B., Wang, Y., Qian, P.Y. (2016) Sensitivity and correlation of hypervariable regions in 16S rRNA genes in phylogenetic analysis. *BMC Bioinformatics* **17**, 135.

Yao, S., Ni, J., Ma, T., Li, C. (2013) Heterotrophic nitrification and aerobic denitrification at low temperature by a newly isolated bacterium, acinetobacter sp. HA2. *Bioresource Technology* **139**: 80-6.

Yoon, S.H., Kim, H.S., Yeom, I.T. (2004) The optimum operational condition of membrane bioreactor (MBR): Cost estimation of aeration and sludge treatment. *Water Research* **38**: 37–46.

Yuan, Q.B., Guo, M.T., Yang, J. (2014) Monitoring and assessing the impact of wastewater treatment on release of both antibiotic resistant bacteria and their typical genes in a Chinese municipal wastewater treatment plant. *Environ Sci Process Impacts* **16**(8): 1930–7.

Yue, C. (2017) Phosphorus recovery from a membrane enhanced biological phosphorus removal (MEBPR) process. Ph.D. Dissertation. Department of Civil Engineering, University of British Columbia, Vancouver, Canada.

You, S.J. & Sue, W.M. (2009) Filamentous bacteria in a foaming membrane bioreactor. *Journal of Membrane Science* **342**: 42−9.

Zaikova, E., Walsh, D.A., Stilwell, C.P., Mohn, W.W., Tortell, P.D., Hallam, S.J. (2010) Microbial community dynamics in a seasonally anoxic fjord: Saanich inlet, British Columbia. *Environmental Microbiology* **12**(1): 172-91.

Zhang, Z. & Hall, E.R. (2006) Heterotrophic kinetic parameter estimation for enhanced biological phosphorus removal processes operated in conventional and membrane assisted modes. *Water Quality Research Journal of Canada* **41**(1): 72-83.

Zhang, Y., Marrs, C.F., Simon, C., Xi, C. (2009) Wastewater treatment contributes to selective increase of antibiotic resistance among Acinetobacter spp. *Sci Total Environ* **407**(12): 3702–6.

Zhang, Y., Zhuang, Y., Geng, J., Ren, H., Zhang, Y., Ding, L., Xu, K. (2015a) Inactivation of antibiotic resistance genes in municipal wastewater effluent by chlorination and sequential UV/chlorination disinfection. *Sci Total Environ* **512-513**: 125–32.

Zhang, S., Han, B., Gu, J., Wang, C., Wang, P., Ma, Y., et al. (2015b) Fate of antibiotic resistant cultivable heterotrophic bacteria and antibiotic resistance genes in wastewater treatment processes. Cheosphere **135**: 138-45.

Zhang, X-X. & Zhang, T. (2011) Occurrence, abundance, and diversity of tetracycline resistance genes in 15 sewage treatment plants across China and other global locations. *Environ. Sci. Technol* **45**: 2598–2604.

Zhang, T., Zhang, X-X., Ye, L. (2011) Plasmid metagenome reveals high levels of antibiotic resistance genes and mobile genetic elements in activated sludge. *Plos One* **6**(10): e26041.

Zhong L., Cheng, Q., Tian, X., Zhao, L., Qin, Z. (2010) Characterization of the replication, transfer, and plasmid/lytic phage cycle of the Streptomyces plasmid-phage pZL12. *Journal of Bacteriology* **192**(4): 3747–54.

Zhou, N.A., Lutovsky, A.C., Andaker, G.L., Gough, H.L., Ferguson, J.F. (2013) Cultivation and characterization of bacterial isolates capable of degrading pharmaceutical and personal care products for improved removal in activated sludge wastewater treatment. *Biodegradation* **24**: 813–27.

Zimbro M.J., Power, S.M., Miller, G.E. et al. (2009) Difco and BBL manual: Manual of microbiological culture media. Second Edition, Becton, Dickinson and Company, Sparks, MD.

Zuthi, M.F.R., Guo, W.S., Ngo, H.H., Nghiem, L.D., Hai, F.I. (2013) Enhanced biological phosphorus removal and its modeling for the activated sludge and membrane bioreactor processes. *Bioresource Technology* **139**: 363-74.

APPENDIX A: SECTION 3.1 SUPPLEMENTARY MATERIAL

Table A.1 Antimicrobial susceptibility profiles of influent and membrane-treated effluent SMX^r strains (n=120)[a]

N0.	Strain	Sample	Replicate	AMC 30 (µg/disk)	CM 30 (µg/disk)	TET 5 (µg/disk)	CIP 5 (µg/disk)	TMP 5 (µg/disk)	K 30 (µg/disk)
1	MH-1	Influent	1						
2	MH-2	Influent	1						
3	MH-3	Influent	1		•	•		•	
4	MH-4	Influent	1						
5	MH-5	Influent	1						
6	MH-6	Influent	1			•		•	
7	MH-7	Influent	1						
8	MH-8	Influent	1						
9	MH-9	Influent	1						
10	MH-10	Influent	1			•			
11	MH-11	Influent	2			•		•	•
12	MH-12	Influent	2						
13	MH-13	Influent	2						
14	MH-14	Influent	2			•		•	
15	MH-15	Influent	2						
16	MH-16	Influent	2						
17	MH-17	Influent	2						
18	MH-18	Influent	2						
19	MH-19	Influent	2	•					
20	MH-20	Influent	2		•			•	
21	MH-21	Influent	3						
22	MH-22	Influent	3						
23	MH-23	Influent	3						
24	MH-24	Influent	3			•		•	
25	MH-25	Influent	3						
26	MH-26	Influent	3			•	•		•
27	MH-27	Influent	3						
28	MH-28	Influent	3						
29	MH-29	Influent	3			•			
30	MH-30	Influent	3			•			
31	R2A-1	Influent	1						
32	R2A-2	Influent	1						
33	R2A-3	Influent	1			•	•	•	
34	R2A-4	Influent	1						
35	R2A-5	Influent	1						

N0.	Strain	Sample	Replicate	AMC 30 (µg/disk)	CM 30 (µg/disk)	TET 5 (µg/disk)	CIP 5 (µg/disk)	TMP 5 (µg/disk)	K 30 (µg/disk)
36	R2A-6	Influent	1					•	
37	R2A-7	Influent	1						
38	R2A-8	Influent	1						
39	R2A-9	Influent	1						
40	R2A-10	Influent	1						
41	R2A-11	Influent	2					•	
42	R2A-12	Influent	2						
43	R2A-13	Influent	2						
44	R2A-14	Influent	2						
45	R2A-15	Influent	2						
46	R2A-16	Influent	2						
47	R2A-17	Influent	2						
48	R2A-18	Influent	2						
49	R2A-19	Influent	2						
50	R2A-20	Influent	2				•	•	
51	R2A-21	Influent	3						
52	R2A-22	Influent	3						
53	R2A-23	Influent	3						
54	R2A-24	Influent	3						
55	R2A-25	Influent	3			•			
56	R2A-26	Influent	3						
57	R2A-27	Influent	3						
58	R2A-28	Influent	3						
59	R2A-29	Influent	3						
60	R2A-30	Influent	3						
61	MH-1	Effluent	1						
62	MH-2	Effluent	1					•	
63	MH-3	Effluent	1						
64	MH-4	Effluent	1						
65	MH-5	Effluent	1			•		•	
66	MH-6	Effluent	1			•			
67	MH-7	Effluent	1						
68	MH-8	Effluent	1						
69	MH-9	Effluent	1			•			
70	MH-10	Effluent	1		•	•			
71	MH-11	Effluent	2			•	•	•	•
72	MH-12	Effluent	2						
73	MH-13	Effluent	2						
74	MH-14	Effluent	2			•		•	
75	MH-15	Effluent	2						
76	MH-16	Effluent	2						
77	MH-17	Effluent	2		•	•	•	•	

N0.	Strain	Sample	Replicate	AMC 30 (µg/disk)	CM 30 (µg/disk)	TET 5 (µg/disk)	CIP 5 (µg/disk)	TMP 5 (µg/disk)	K 30 (µg/disk)
78	MH-18	Effluent	2						
79	MH-19	Effluent	2						
80	MH-20	Effluent	2					•	
81	MH-21	Effluent	3		•			•	
82	MH-22	Effluent	3					•	
83	MH-23	Effluent	3						
84	MH-24	Effluent	3						
85	MH-25	Effluent	3			•		•	
86	MH-26	Effluent	3						
87	MH-27	Effluent	3						
88	MH-28	Effluent	3						
89	MH-29	Effluent	3					•	
90	MH-30	Effluent	3	•	•			•	
91	R2A-1	Effluent	1						
92	R2A-2	Effluent	1						
93	R2A-3	Effluent	1			•		•	•
94	R2A-4	Effluent	1						
95	R2A-5	Effluent	1						
96	R2A-6	Effluent	1	•	•	•		•	
97	R2A-7	Effluent	1						
98	R2A-8	Effluent	1						
99	R2A-9	Effluent	1						
100	R2A-10	Effluent	1			•		•	
101	R2A-11	Effluent	2						
102	R2A-12	Effluent	2						
103	R2A-13	Effluent	2						
104	R2A-14	Effluent	2						
105	R2A-15	Effluent	2		•	•			•
106	R2A-16	Effluent	2						
107	R2A-17	Effluent	2						
108	R2A-18	Effluent	2						
109	R2A-19	Effluent	2						
110	R2A-20	Effluent	2					•	
111	R2A-21	Effluent	3						
112	R2A-22	Effluent	3						
113	R2A-23	Effluent	3			•		•	•
114	R2A-24	Effluent	3			•		•	
115	R2A-25	Effluent	3						
116	R2A-26	Effluent	3						
117	R2A-27	Effluent	3						
118	R2A-28	Effluent	3						

N0.	Strain	Sample	Replicate	AMC 30 (µg/disk)	CM 30 (µg/disk)	TET 5 (µg/disk)	CIP 5 (µg/disk)	TMP 5 (µg/disk)	K 30 (µg/disk)
119	R2A-29	Effluent	3					•	
120	R2A-30	Effluent	3	•				•	

[a] Filled black circles indicate resistance (diameter of the zone of inhibition ≤ one centimeter); the absence of a circle mark indicates a larger inhibition zone.

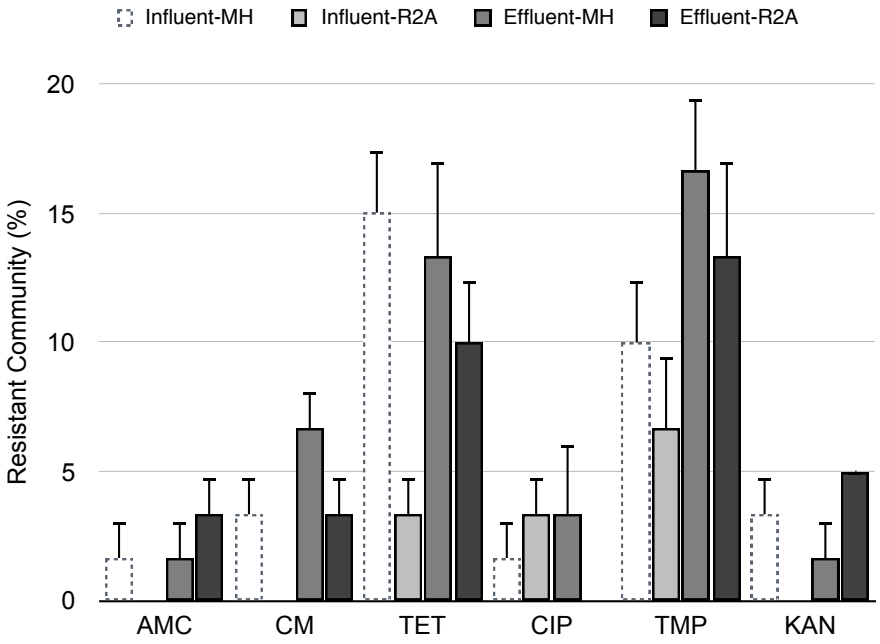
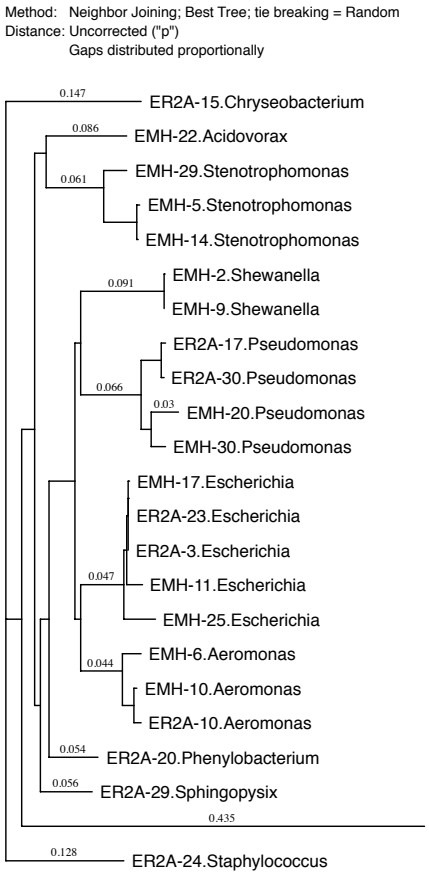


Figure A.1 Comparison of the percentage of ARB between MH and R2A SMX^r tested strains (n=120)



Strain	Species	bp	Identity	AMC (30)	CM (30)	TET (5)	CIP (5)	TMP (5)	K (30)
R2A-15	*Chryseobacterium hispalense*	1201	99%		•	•			•
MH-22	*Acidovorax delafieldii*	1324	99%					•	
MH-29	*Stenotrophomonas acidaminiphia*	1402	99%					•	
MH-5	*Stenotrophomonas sp.*	1416	99%			•		•	
MH-14	*Stenotrophomonas maltophilia*	1415	99%			•		•	
MH-2	*Shewanella xiamenesis*	1420	99%					•	
MH-9	*Shewanella xiamenesis*	1373	99%			•			
R2A-17	*Pseudomonas chloraraphis*	1373	99%						
R2A-30	*Pseudomonas putida*	1352	99%	•				•	
MH-20	*Pseudomonas Putida*	526	98%					•	
MH-30	*Pseudomonas helmanticensis*	1387	99%	•	•			•	
MH-17	*Escherichia coli*	1399	99%		•	•	•	•	
R2A-23	*Escherichia coli*	1040	98%			•		•	•
R2A-3	*Escherichia coli*	918	99%			•		•	•
MH-11	*Escherichia coli*	1365	99%			•	•	•	•
MH-25	*Escherichia coli*	1370	99%			•		•	
MH-6	*Aeromonas hydrophila*	1042	99%			•			
MH-10	*Aeromonas hydrophila*	1382	98%		•	•			
R2A-10	*Aeromonas hydrophila*	1401	99%			•		•	
R2A-20	*Phenylobacterium conjuctum*	1325	99%					•	
R2A-29	*Sphingopysix taejonensis*	1353	99%					•	
MH-21	*Comamonas odontotermitis*	1055	99%		•			•	
R2A-24	*Staphylococcus equorum*	1386	100%			•		•	

Figure A.2 Phylogenetic tree of 16S-sequenced SMX^r effluent bacterial isolates conferring resistance to a minimum of two antibiotics

Strain	Species	bp	Identity	AMC (30)	CM (30)	TET (5)	CIP (5)	TMP (5)	K (30)
MH-20	*Comamonas odontotermititis*	401	98%		•			•	
MH-29	*Burkholderia sp.*	644	97%			•			
R2A-11	*Stenotrophomonas sp.*	796	100%					•	
MH-11	*Escherichia sp.*	993	99%			•		•	•
MH-26	*Escherichia coli*	591	100%			•	•		•
MH-3	*Escherichia coli*	711	99%		•	•		•	
MH-14	*Escherichia coli*	610	100%			•		•	
MH-30	*Escherichia coli*	925	99%			•			
MH-6	*Escherichia coli*	761	98%			•		•	
R2A-3	*Escherichia coli*	879	99%			•	•	•	
MH-19	*Enterobacter sp.*	267	98%	•					
R2A-6	*Citrobacter sp.*	1135	98%					•	
MH-10	*Aeromonas hydrophila*	480	99%			•			
R2A-25	*Acinetobacter haemolyticus*	468	99%			•			
MH-24	*Uruburuella suis*	1744	96%			•		•	
R2A-20	*Cloacibacterium rupense*	1170	99%				•	•	

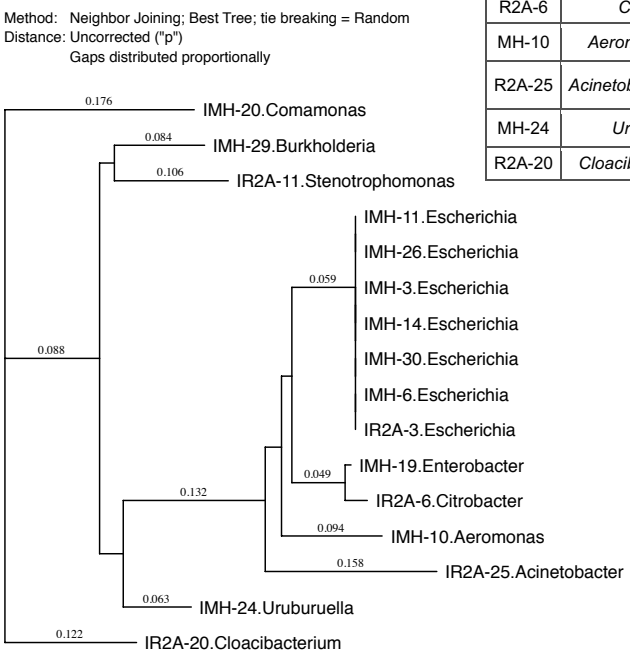


Figure A.3 Phylogenetic tree of 16S-sequenced SMX^r influent bacterial isolates conferring resistance to a minimum of two antibiotics

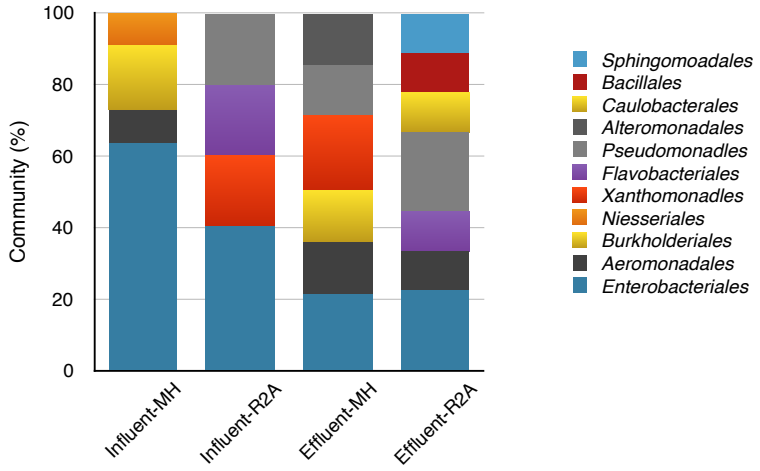


Figure A.4 Composition of influent and membrane effluent SMX^r 16S-sequenced bacterial isolates at the order level (Sampling: January 2014)

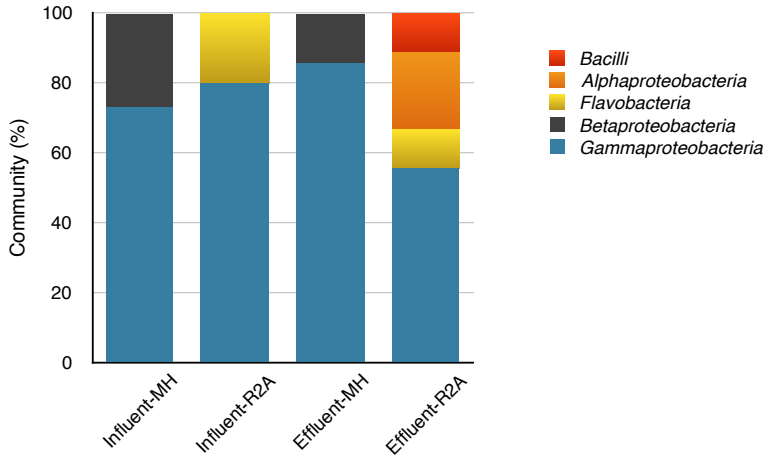


Figure A.5 Composition of influent and membrane effluent SMX^r 16S-sequenced bacterial isolates at the class level (Sampling: January 2014)

Table A.2 A summary of the *sul*1 gene concentrations (normalized to 16S rRNA gene) at different sampling dates and locations in the present study

Sample (Sampling Day)	January 7th, 2014	June 16th, 2014	December 3rd, 2014	January 6th, 2015	February 3rd, 2015
Influent	0.0114 σ=0.0013	0.0097 σ=0.0005	0.0128 σ=0.0016	0.0085 σ=0.0003	0.0056 σ=0.0006
Anaerobic (SRT = 25 days)	-	0.0187 σ=0.0006	-	0.0097 σ=0.0002	0.0221 σ=0.0014
Anoxic (SRT = 25 days)	-	0.0201 σ=0.0017	0.0092 σ=0.0006	-	0.0184 σ=0.0012
Aerobic (SRT = 25 days)	-	-	0.0126 σ=0.0004	0.0071 σ=0.0004	0.0188 σ=0.0022
Effluent (SRT = 25 days)	0.0063 σ=0.0041	0.0198 σ=0.0019	0.0095 σ=0.0005	0.0212 σ=0.0024	0.0118 σ=0.0118
Anaerobic (SRT = 60 days)	-	0.012 σ=0.0005	0.0246 σ=0.0023	0.0174 σ=0.0028	0.0122 σ=0.0008
Anoxic (SRT = 60 days)	-	-	0.0218 σ=0.0018	0.0252 σ=0.0015	0.015 σ=0.0004
Aerobic (SRT = 60 days)	-	0.011 σ=0.0010	0.0225 σ=0.0088	0.0244 σ=0.0015	0.0078 σ=0.0004
Effluent (SRT = 60 days)	-	0.0089 σ=0.0009	0.014 σ=0.0019	0.0178 σ=0.0014	0.0077 σ=0.0007

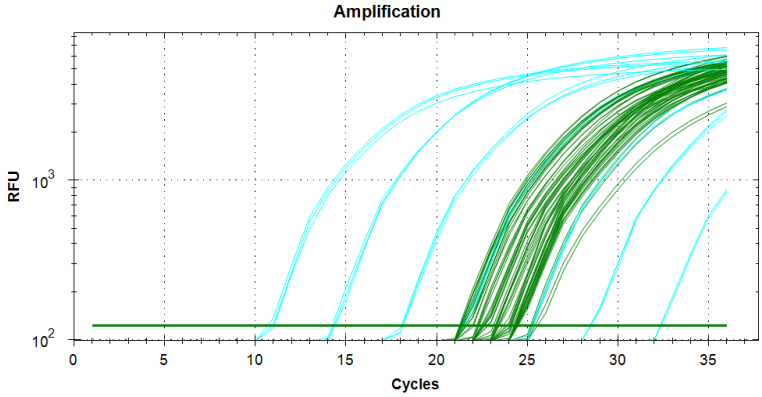


Figure A.6 qPCR amplification curve (*sul*1 gene)

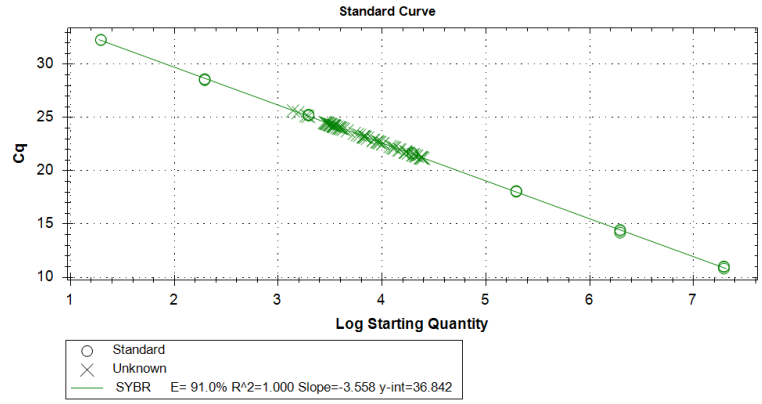


Figure A.7 Standard curve (*sul*1 gene)

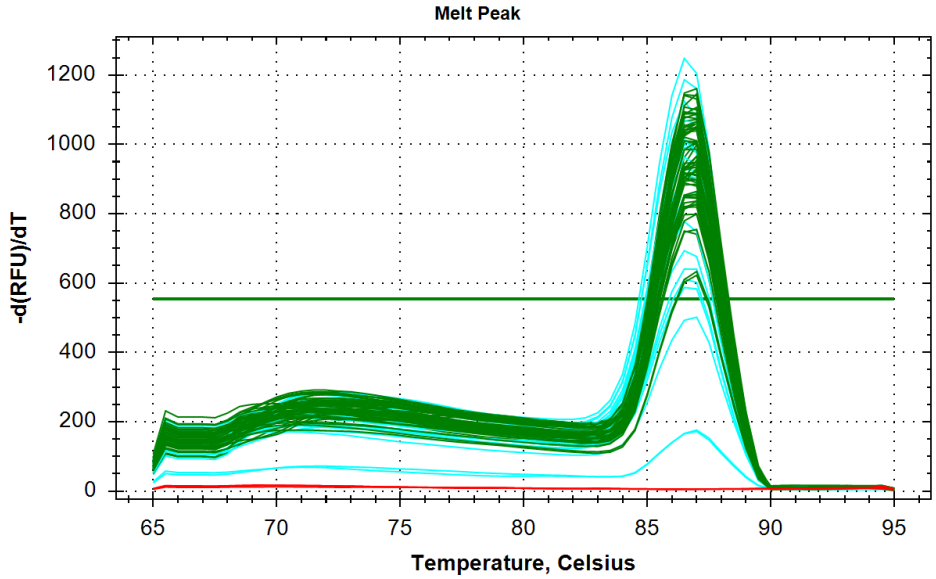


Figure A.8 Melt peak curve (*sul*1 gene)

APPENDIX B: SECTION 3.2 SUPPLEMENTARY MATERIAL

Table B.1 ARB percentage (%) of cultivable total heterotrophic bacteria in influent and parallel MEBPR systems

Sample: Influent	Media: MH					Media: R2A				
Antibiotic	AMX	CIP	SMX	TET	TMP	AMX	CIP	SMX	TET	TMP
Dec 5th, 2014	53.1	0.0	45.9	1.0	22.5	8.4	0.9	12.1	0.9	9.3
Jan 6th, 2015	36.6	0.9	40.6	8.5	21.9	13.6	1.0	14.6	0.0	13.1
Feb 3rd, 2015	43.4	0.7	28.9	5.9	19.7	14.5	2.4	10.8	2.4	7.2
Average	44.4	0.5	38.5	5.1	21.4	12.1	1.4	12.5	1.1	9.9
Standard Deviation	6.8	0.4	7.1	3.1	1.2	2.7	0.7	1.5	1.0	2.4
Standard Error Mean	3.9	0.2	4.1	1.8	0.7	1.5	0.4	0.9	0.6	1.4
Sample: Mixed Liquor (25 day SRT)	Media: MH					Media: R2A				
Antibiotic	AMX	CIP	SMX	TET	TMP	AMX	CIP	SMX	TET	TMP
Dec 5th, 2014	40.8	1.0	49.0	10.2	29.6	24.3	1.0	15.5	7.8	17.5
Jan 6th, 2015	47.3	0.5	51.1	9.8	31.5	18.1	2.5	16.9	6.3	15.6
Feb 3rd, 2015	52.8	0.0	52.4	6.0	34.1	22.6	0.6	14.2	0.0	20.0
Average	47.0	0.5	50.8	8.6	31.7	21.7	1.4	15.5	4.7	17.7
Standard Deviation	4.9	0.4	1.4	1.9	1.9	2.6	0.8	1.1	3.4	1.8
Standard Error Mean	2.8	0.2	0.8	1.1	1.1	1.5	0.5	0.6	1.9	1.0
Sample: Foam (25 day SRT)	Media: MH					Media: R2A				
Antibiotic	AMX	CIP	SMX	TET	TMP	AMX	CIP	SMX	TET	TMP
Dec 5th, 2014	43.2	0.8	52.5	5.9	24.2	23.4	1.1	13.0	2.7	15.8
Jan 6th, 2015	38.9	0.0	37.7	5.7	32.0	27.3	0.6	18.8	1.7	17.6
Feb 3rd, 2015	44.9	0.0	48.1	9.5	32.9	22.5	1.1	11.2	3.4	13.5
Average	42.3	0.3	46.1	7.0	29.7	24.4	0.9	14.3	2.6	15.6
Standard Deviation	2.6	0.4	6.2	1.7	3.9	2.1	0.3	3.2	0.7	1.7
Standard Error Mean	1.5	0.2	3.6	1.0	2.3	1.2	0.1	1.9	0.4	1.0
Sample: Effluent (25 day SRT)	Media: MH					Media: R2A				
Antibiotic	AMX	CIP	SMX	TET	TMP	AMX	CIP	SMX	TET	TMP
Dec 5th, 2014	58.3	1.4	54.2	8.3	29.2	20.7	0.4	25.7	5.1	13.5
Jan 6th, 2015	49.0	0.0	51.7	6.0	35.8	24.4	1.1	14.4	3.3	27.8
Feb 3rd, 2015	51.5	0.5	46.5	2.5	27.2	20.8	1.4	14.0	4.3	14.5
Average	52.9	0.6	50.8	5.6	30.7	22.0	1.0	18.1	4.2	18.6
Standard Deviation	3.9	0.6	3.2	2.4	3.7	1.8	0.4	5.4	0.7	6.5
Standard Error Mean	2.3	0.3	1.8	1.4	2.1	1.0	0.2	3.1	0.4	3.8

Sample: Mixed Liquor (60 day SRT)	Media: MH					Media: R2A				
Antibiotic	**AMX**	**CIP**	**SMX**	**TET**	**TMP**	**AMX**	**CIP**	**SMX**	**TET**	**TMP**
Dec 5th, 2014	52.3	0.3	46.2	7.1	21.5	32.6	0.0	16.3	10.1	16.3
Jan 6th, 2015	47.9	0.0	47.9	6.7	32.2	32.5	5.3	24.6	7.0	24.6
Feb 3rd, 2015	55.9	0.4	52.5	8.8	41.6	26.0	3.1	18.9	7.1	25.0
Average	52.0	0.2	48.9	7.5	31.8	30.3	2.8	19.9	8.1	21.9
Standard Deviation	3.2	0.2	2.7	0.9	8.2	3.1	2.2	3.5	1.4	4.0
Standard Error Mean	1.9	0.1	1.5	0.5	4.7	1.8	1.2	2.0	0.8	2.3
Sample: Foam (60 day SRT)	**Media: MH**					**Media: R2A**				
Antibiotic	**AMX**	**CIP**	**SMX**	**TET**	**TMP**	**AMX**	**CIP**	**SMX**	**TET**	**TMP**
Dec 5th, 2014	48.8	1.8	51.2	9.5	33.4	27.0	0.0	20.4	6.6	26.5
Jan 6th, 2015	44.9	1.4	51.9	11.6	32.4	20.4	6.1	27.6	3.1	31.6
Feb 3rd, 2015	57.7	1.2	47.6	5.9	38.1	29.5	1.6	29.0	4.7	34.2
Average	50.5	1.5	50.2	9.0	34.6	25.7	2.6	25.7	4.8	30.8
Standard Deviation	5.4	0.3	1.9	2.3	2.5	3.9	2.6	3.8	1.5	3.2
Standard Error Mean	3.1	0.2	1.1	1.3	1.4	2.2	1.5	2.2	0.8	1.8
Sample: Effluent (60 day SRT)	**Media: MH**					**Media: R2A**				
Antibiotic	**AMX**	**CIP**	**SMX**	**TET**	**TMP**	**AMX**	**CIP**	**SMX**	**TET**	**TMP**
Dec 5th, 2014	47.8	0.8	49.0	11.1	25.7	36.8	2.6	18.4	7.4	27.9
Jan 6th, 2015	51.1	2.3	51.7	9.2	33.9	29.5	0.0	23.1	6.4	23.1
Feb 3rd, 2015	57.9	5.6	49.6	9.9	39.7	31.4	2.9	26.2	5.8	29.1
Average	52.3	2.9	50.1	10.1	33.1	32.6	1.8	22.6	6.5	26.7
Standard Deviation	4.2	2.0	1.2	0.8	5.7	3.1	1.3	3.2	0.6	2.6
Standard Error Mean	2.4	1.1	0.7	0.4	3.3	1.8	0.8	1.8	0.4	1.5

Table B.2 Statistically significant results comparying two means of MH populations (P ≤ 0.05)

Sample		Influent (MH)					Mixed Liquor (25 day SRT) (MH)					Mixed Liquor (60 day SRT) (MH)					Effluent (25 day SRT) (MH)					Effluent (60 day SRT) (MH)				
Antibiotic		SMX	TMP	CIP	AMX	TET	SMX	TMP	CIP	AMX	TET	SMX	TMP	CIP	AMX	TET	SMX	TMP	CIP	AMX	TET	SMX	TMP	CIP	AMX	TET
Influent (MH)	SMX	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	TMP	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	CIP	S	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	AMX	N	S	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	TET	S	S	S*	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mixed Liquor (25 day SRT) (MH)	SMX	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	TMP	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	CIP	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	AMX	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	TET	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mixed Liquor (60 day SRT) (MH)	SMX	S*	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	TMP	-	S*	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	CIP	-	-	N	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	AMX	-	-	-	N	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	TET	-	-	-	-	N	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Effluent (25 day SRT) (MH)	SMX	S*	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	TMP	-	S	-	-	-	-	N	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-
	CIP	-	-	N	-	-	-	-	N	-	-	-	-	-	-	-	S	S	-	-	-	-	-	-	-	-
	AMX	-	-	-	N	-	-	-	-	N	-	-	-	-	-	-	N	S	S	-	-	-	-	-	-	-
	TET	-	-	-	-	N	-	-	-	-	N	-	-	-	-	-	S	S	S	S	-	-	-	-	-	-
Effluent (60 day SRT) (MH)	SMX	S*	-	-	-	-	-	-	-	-	-	N	-	-	-	-	N	-	-	-	-	-	-	-	-	-
	TMP	-	S	-	-	-	-	-	-	-	-	-	N	-	-	-	-	N	-	-	-	S	-	-	-	-
	CIP	-	-	S*	-	-	-	-	-	-	-	-	-	S	-	-	-	-	S*	-	-	S	S	-	-	-
	AMX	-	-	-	N	-	-	-	-	-	-	-	-	-	N	-	-	-	-	N	-	N	S	S	-	-
	TET	-	-	-	-	S*	-	-	-	-	-	-	-	-	-	S	-	-	-	-	S	S	S	S	S	-
Foam (25 day SRT) (MH)	SMX	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	TMP	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	CIP	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	AMX	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	TET	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Sample		Influent (MH)					Mixed Liquor (25 day SRT) (MH)					Mixed Liquor (60 day SRT) (MH)					Effluent (25 day SRT) (MH)					Effluent (60 day SRT) (MH)				
Antibiotic		SMX	TMP	CIP	AMX	TET	SMX	TMP	CIP	AMX	TET	SMX	TMP	CIP	AMX	TET	SMX	TMP	CIP	AMX	TET	SMX	TMP	CIP	AMX	TET
Foam (60 day SRT) (MH)	SMX	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	TMP	-	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-
	CIP	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-
	AMX	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-
	TET	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-

S: statistically significant, N: not-significant, -:not analyzed

Table B.3 Statistically significant results comparing two means of R2A populations (P ≤ 0.05)

Sample		Influent (R2A)					Mixed Liquor (25 day SRT) (R2A)					Mixed Liquor (60 day SRT) (R2A)					Effluent (25 day SRT) (R2A)					Effluent (60 day SRT) (R2A)				
Antibiotic		SMX	TMP	CIP	AMX	TET	SMX	TMP	CIP	AMX	TET	SMX	TMP	CIP	AMX	TET	SMX	TMP	CIP	AMX	TET	SMX	TMP	CIP	AMX	TET
Influent (R2A)	SMX	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	TMP	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	CIP	S	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	AMX	N	N	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	TET	S	S	N	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mixed Liquor (25 day SRT) (R2A)	SMX	S*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	TMP	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	CIP	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	AMX	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	TET	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mixed Liquor (60 day SRT) (R2A)	SMX	S	-	-	-	-	S*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	TMP	-	S	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	CIP	-	-	N	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	AMX	-	-	-	S	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	TET	-	-	-	-	S	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Effluent (25 day SRT) (R2A)	SMX	N	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	TMP	-	S*	-	-	-	-	N	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-
	CIP	-	-	N	-	-	-	-	N	-	-	-	-	-	-	-	S	S	-	-	-	-	-	-	-	-
	AMX	-	-	-	S	-	-	-	-	N	-	-	-	-	-	-	N	N	S	-	-	-	-	-	-	-
	TET	-	-	-	-	S	-	-	-	-	N	-	-	-	-	-	S	S	S	S	-	-	-	-	-	-
Effluent (60 day SRT) (R2A)	SMX	S	-	-	-	-	-	-	-	-	-	N	-	-	-	-	N	-	-	-	-	-	-	-	-	-
	TMP	-	S	-	-	-	-	-	-	-	-	-	N	-	-	-	-	S*	-	-	-	N	-	-	-	-
	CIP	-	-	N	-	-	-	-	-	-	-	-	-	N	-	-	-	-	N	-	-	S	S	-	-	-
	AMX	-	-	-	S	-	-	-	-	-	-	-	-	-	N	-	-	-	-	S	-	S	S*	S	-	-
	TET	-	-	-	-	S	-	-	-	-	-	-	-	-	-	N	-	-	-	-	S	S	S	S	S	-
Foam (25 day SRT) (R2A)	SMX	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	TMP	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	CIP	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	AMX	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	TET	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Sample		Influent (R2A)					Mixed Liquor (25 day SRT) (R2A)					Mixed Liquor (60 day SRT) (R2A)					Effluent (25 day SRT) (R2A)					Effluent (60 day SRT) (R2A)				
Antibiotic		SMX	TMP	CIP	AMX	TET	SMX	TMP	CIP	AMX	TET	SMX	TMP	CIP	AMX	TET	SMX	TMP	CIP	AMX	TET	SMX	TMP	CIP	AMX	TET
Foam (60 day SRT) (R2A)	SMX	-	-	-	-	-	-	-	-	-	-	S*	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	TMP	-	-	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-
	CIP	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-
	AMX	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-
	TET	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S*	-	-	-	-	-	-	-	-	-	-

S: statistically significant at 5% significance level, N: not-significant, -:not analyzed, S*: statistically significant at 10% significance level

Table B.4 Statistically significant results comparying MH and R2A population mean values (P ≤ 0.05)

Sample		Influent (MH)					Mixed Liquor (25 day SRT) (MH)					Mixed Liquor (60 day SRT) (MH)					Effluent (25 day SRT) (MH)					Effluent (60 day SRT) (MH)				
Antibiotic		SMX	TMP	CIP	AMX	TET	SMX	TMP	CIP	AMX	TET	SMX	TMP	CIP	AMX	TET	SMX	TMP	CIP	AMX	TET	SMX	TMP	CIP	AMX	TET
Influent (R2A)	SMX	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	TMP	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	CIP	-	-	S*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	AMX	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	TET	-	-	-	-	S*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mixed Liquor (25 day SRT) (R2A)	SMX	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	TMP	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	CIP	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	AMX	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	TET	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mixed Liquor (60 day SRT) (R2A)	SMX	-	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	TMP	-	-	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-	-	-
	CIP	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-	-	-
	AMX	-	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-	-	-
	TET	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-	-	-
Effluent (25 day SRT) (R2A)	SMX	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-	-	-	-
	TMP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S*	-	-	-	-	-	-	-	-
	CIP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-
	AMX	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	-
	TET	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-	-	-	-
Effluent (60 day SRT) (R2A)	SMX	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-	-	-
	TMP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-	-
	CIP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	N	-	-
	AMX	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	-
	TET	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S

S: statistically significant at 5% significance level, N: not-significant, -:not analyzed, S*: statistically significant at 10% significance

Table B.5 A summary of colony counts of total cultivable heterotrophic bacteria in influent and MEBPR environments (Sampling: December 2014, January and February 2015)

Sample	Media	Average heterotrophic cell counts (SRT = 25 days) (n=27)	Average heterotrophic cell counts (SRT = 60 days) (n=27)
Influent	MH	1.1×10^{6} ($\sigma=2.2\times10^{5}$)	
	R2A	4.3×10^{6} ($\sigma=8.9\times10^{5}$)	
Aerobic mixed liquor	MH	6.7×10^{6} ($\sigma=7.5\times10^{5}$)	1.3×10^{7} ($\sigma=2.9\times10^{6}$)
	R2A	3.3×10^{7} ($\sigma=2.3\times10^{6}$)	6×10^{7} ($\sigma=7.6\times10^{6}$)
Treated effluent	MH	505 (σ=655)	63 (σ=15)
	R2A	2.2×10^{3} ($\sigma=2.7\times10^{3}$)	317(σ=66)

Table B.6 A summary of the occurrence of sulfonamide resistance genes (*sul*1 and *sul*2) in MH and R2A 16S-sequenced treated effluent bacterial isolates

Species	Identity (%)	Strain	Resistance phenotype	SMX Resistance Genes	Class 1 Integron Gene
Escherichia coli	97	MH-I3	AMX,SMX,TET,TMP	*sul*1	*int*1
Aeromonas hydrophila	100	MH-B17	AMX,SMX,TET	*sul*1, *sul*2	-
Klebsiella pneumoniae	99	MH-B23	SMX,TET,TMP	*sul*2	-
Bacillus cereus	99	MH-C7	AMX,TMP,SMX	*sul*1	-
Enterobacter cloacae	99	MH-C13	AMX,SMX	*sul*1	*int*1
Aeromonas hydrophila	99	MH-N23	AMX,SMX	*sul*2	-
Pseudomonas aeruginosa	99	MH-F15	TMP,SMX	*sul*2	-
Comamonas testosteroni	99	R2A-E7	SMX,TMP	*sul*1, *sul*2	*int*1
Acinetobacter sp.	99	R2A-I40	TMP,SMX	*sul*2	-
Stenotrophomonas maltophilia	99	R2A-N5	TET,SMX,AMX	*sul*1	-
Aeromonas allosaccarophila	97	R2A-F17	AMX,SMX,TET	*sul*2	-
Deinococcus arenae	100	R2A-P9	TMP,SMX	*sul*1	*int*1
Aeromonas hydrophila	100	R2A-E19	AMX,SMX	*sul*1	*int*1
Raoultella planticola	99	R2A-G11	SMX	*sul*1	-
Chryseobacterium taiwanense	98	R2A-L21	SMX	*sul*2	-

APPENDIX C: SECTION 3.3 SUPPLEMENTARY MATERIAL

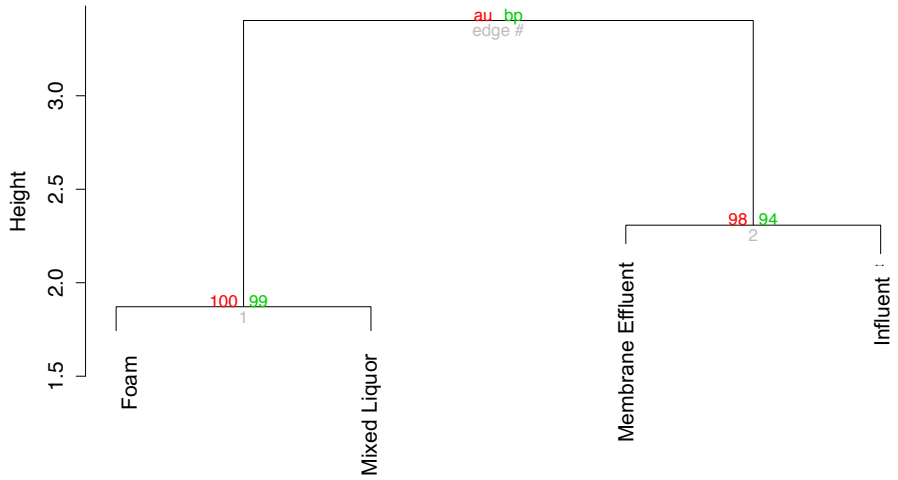


Figure C.1 Cluster dendrogram of taxa with relative abundance < 0.1% in influent and MEBPR environments (SRT = 25 days)

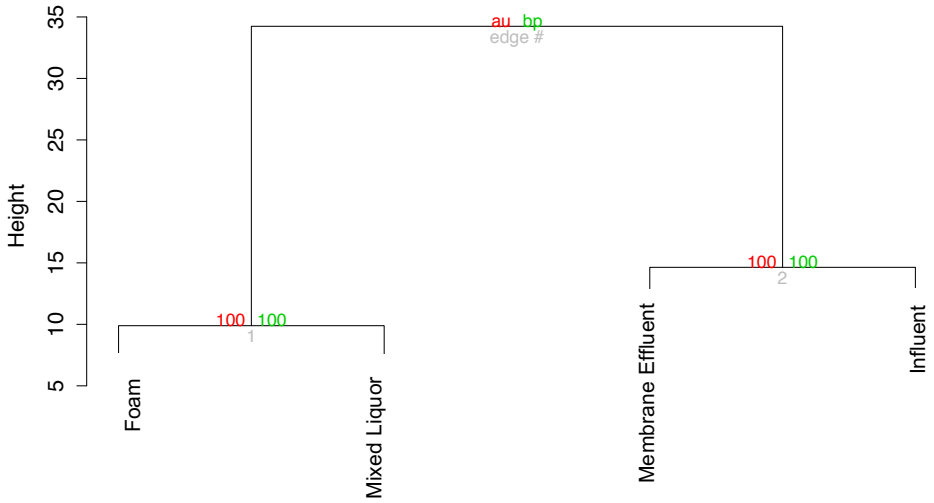


Figure C.2 Cluster dendrogram of taxa (0.1% < relative abundance < 1%) in influent and MEBPR environments (SRT = 25 days)

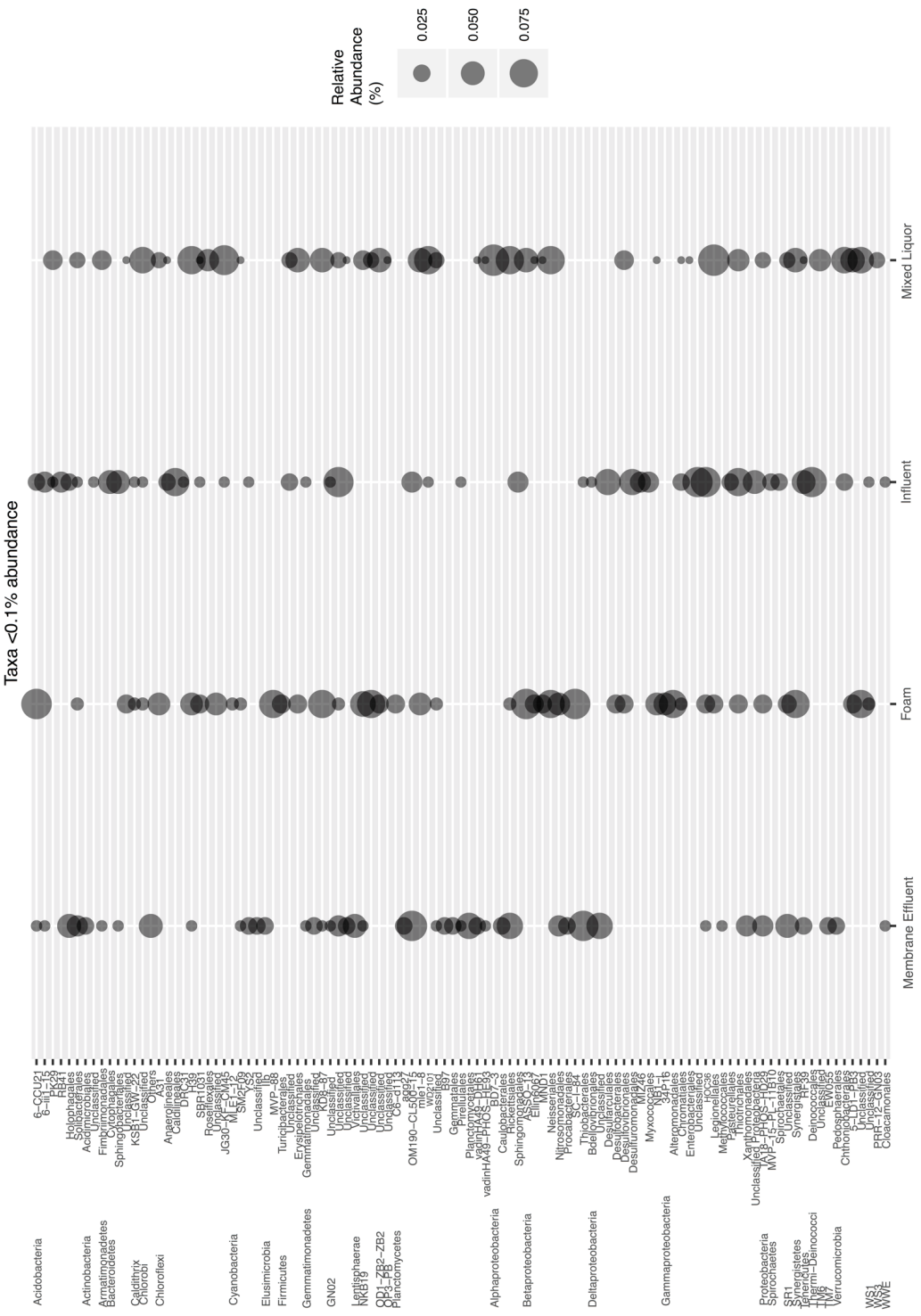


Figure C.3 Taxonomic composition of OTUs with relative abundance < 0.1% in influent and MEBPR environments (SRT = 25 days)

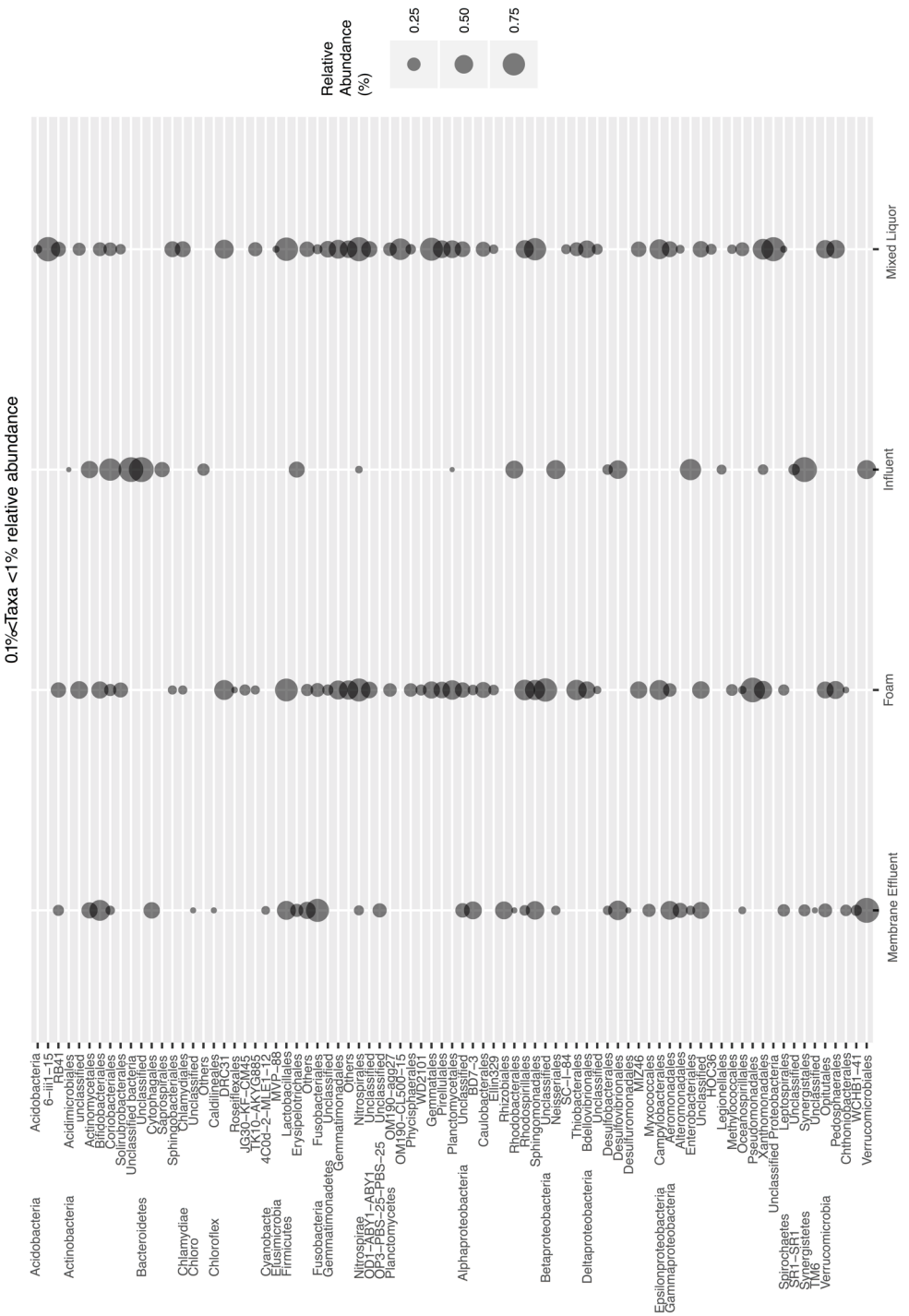


Figure C.4 Taxonomic composition of OTUs (0.1% < relative abundance < 1%) in influent and MEBPR environments (SRT = 25 days)

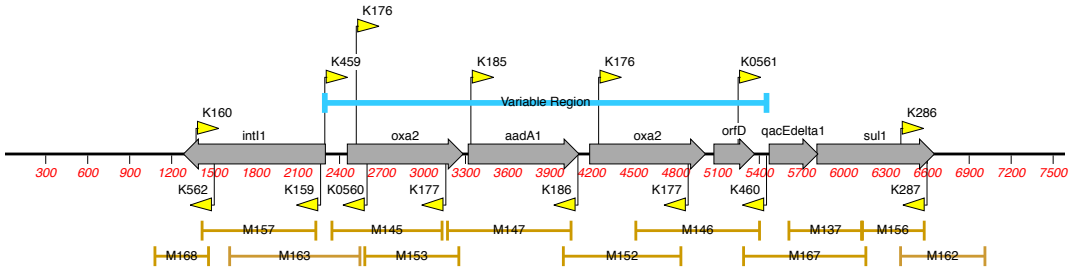


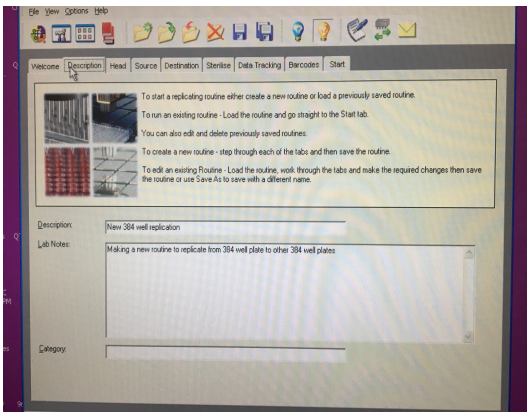
Figure C.5 Detailed sequencing steps involved in identification of the class 1 integron in EPI300: pF3.18 (foam fosmid library CARB^r clone)[*]

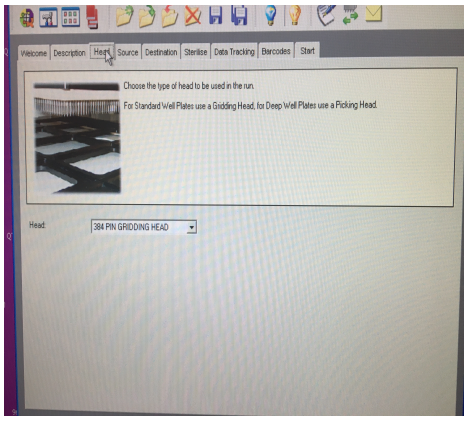
[*] Regions M145, M146, M156, M162, M163, M167, M168 and M147 were identified by sequencing the pF3.18 using primers K459, K460, K287, K286, K560, K561, K562 and K186 respectively. Region M137 was identified by sequencing the Tn5 library clone (SMX-sensitive *E. coli* EPI300:pF3.18:TN5.N3) using primer KAN-2-FP-1. The Tn5 transposon was inserted at 325 bp away from the start site of the *sul*1 gene. Region M157 was identified by PCR using primers K160 and K159 and sequencing the PCR products using primer K159. Region M153 were identified by PCR using primers K459 and K186 and sequencing the PCR products using primer K176. Region M152 was identified by PCR using primers K185 and K177 and sequencing the PCR products using primer K177. It should be noted that the procedure for construction of the Tn5 library was provided in Section 2.4.2.7.

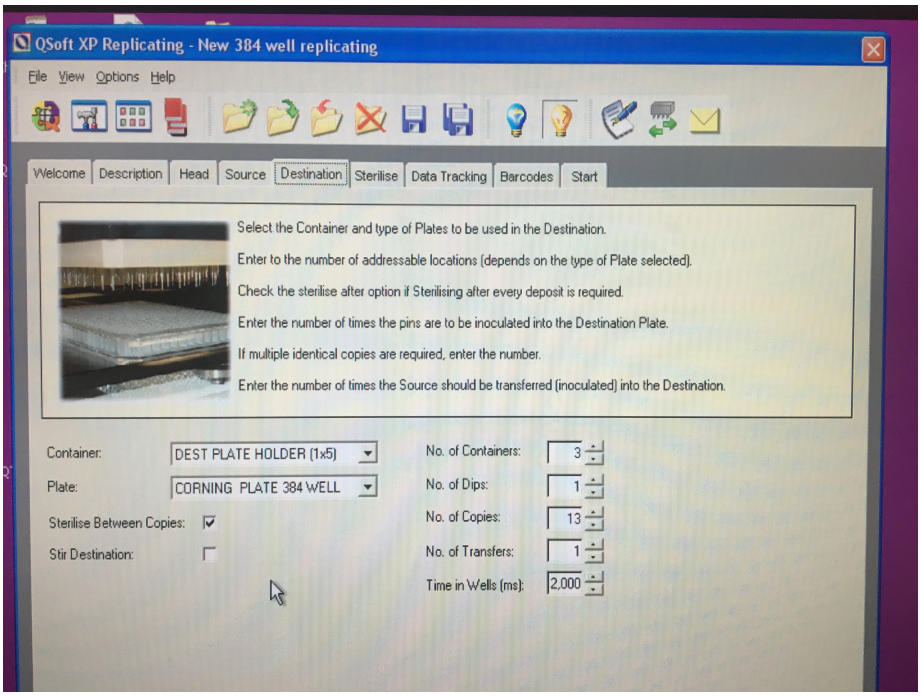
APPENDIX D: PHOTOGRAPHS

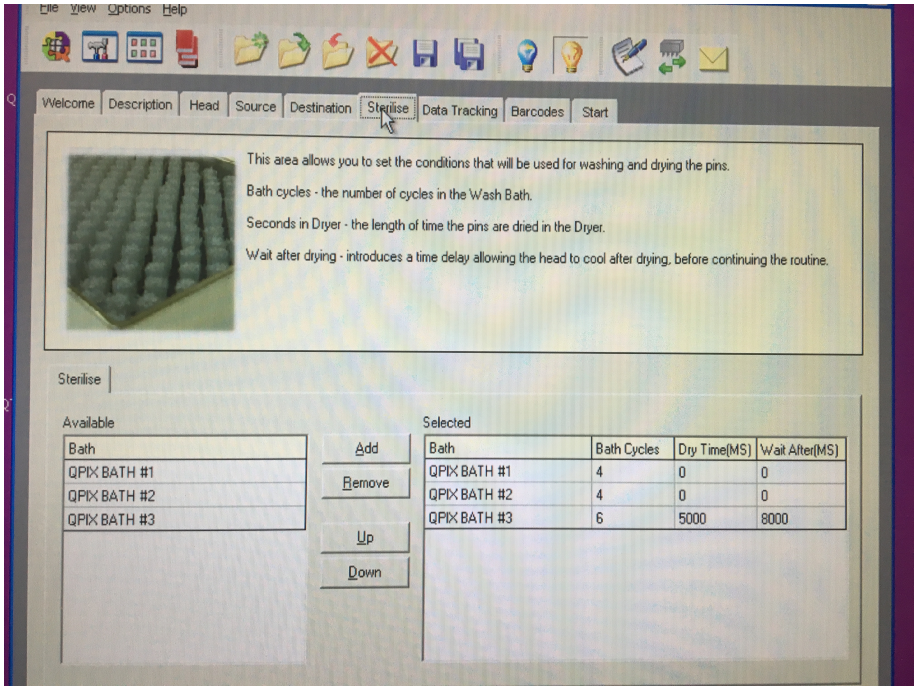
Top: Picture of the QPix2 Robotic colony picker

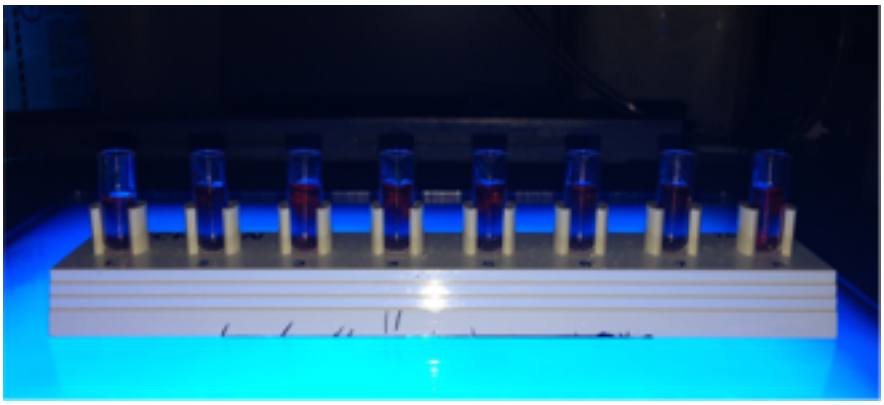
Bottom and next page: The protocol of replicating 384 well plates by QPix2 Robotic colony picker





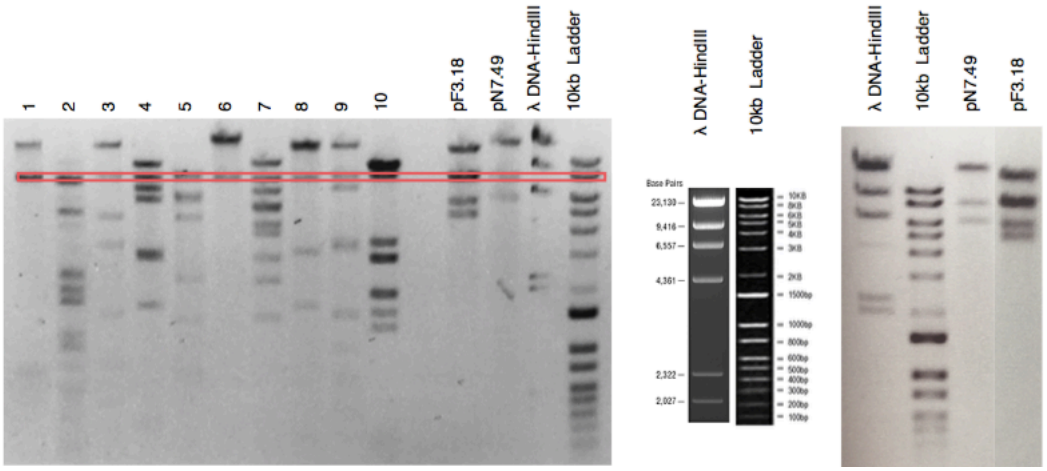

QSoft XP Replicating - New 384 well replicating
File View Options Help
Welcome Description Head Source Destination Sterilise Data Tracking Barcodes Start
Select the Container and type of Plates to be used in the Destination.
Enter to the number of addressable locations (depends on the type of Plate selected).
Check the sterilise after option if Sterilising after every deposit is required.
Enter the number of times the pins are to be inoculated into the Destination Plate.
If multiple identical copies are required, enter the number.
Enter the number of times the Source should be transferred (inoculated) into the Destination.
Container: DEST PLATE HOLDER (1x5)
Plate: CORNING PLATE 384 WELL
Sterilise Between Copies:
Stir Destination:
No. of Containers: 3
No. of Dips: 1
No. of Copies: 13
No. of Transfers: 1
Time in Wells (ms): 2,000


File View Options Help
Welcome Description Head Source Destination Sterilise Data Tracking Barcodes Start
This area allows you to set the conditions that will be used for washing and drying the pins.
Bath cycles - the number of cycles in the Wash Bath.
Seconds in Dryer - the length of time the pins are dried in the Dryer.
Wait after drying - introduces a time delay allowing the head to cool after drying, before continuing the routine.
Sterilise
Available
Bath
QPIX BATH #1
QPIX BATH #2
QPIX BATH #3
Add
Remove
Up
Down
Selected
Bath Bath Cycles Dry Time(MS) Wait After(MS)
QPIX BATH #1 4 0 0
QPIX BATH #2 4 0 0
QPIX BATH #3 6 5000 8000



DNA band illuminated by blue light after CsCl-EtBr gradient centrifugation

(preparation of foam fosmid library)



Foam fosmid clones digested with BamHI restriction enzyme

As observed, each clone had a different size of insert DNA as well as a different pattern of

digestion showing that the library was constructed from cloning unique and different pieces of foam DNA. The common band in all the double digested fosmids is a band of DNA with an 8.1 Kb size (as compared by the ladder) which corresponds to 8.106 kb of pCC2FOS vector. It should be noted that pCC2FOS vector contains two restrictions sites for BamHI at 353 and 407 bp. The insert DNA was ligated at Eco72 I site at 382 bp.