DEVELOPMENT AND REMOVAL OF ANTIBIOTIC RESISTANCE GENES

by

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To my parents, Baoshu Song and Mingyi Wang,
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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATION

ARG	Antibiotic resistant gene
B. cereus	*Bacillus cereus*
CFU	Colony forming unit
CNT	Carbon nanotube
DBP	Disinfections byproducts
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
E. coli	*Escherichia coli*
ere	Erythromycin esterification
erm	Erythromycin ribosome methylation
FISH	Fluorescent *in situ* hybridization
HGT	Horizontal gene transfer
LB	Lysogeny broth
MIC	Minimum inhibitory concentration
MLS_B	Macrolide-lincosamide-streptogramin B
MSC	Minimal selective concentration
MWCNT	Multi-walled carbon nanotube
NOM	Natural organic matter
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
qPCR	quantitative polymerase chain reaction
VBNC	Viable but nonculturable
WWTP	Wastewater treatment plant

ABSTRACT

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Antibiotics have been widely used to treat bacterial diseases since the 1940s. However, the benefits offered by antibiotics have gradually faded due to the increased occurrence and frequency of antibiotic resistance. The widespread use of antibiotics has driven selection for resistance in bacteria and is becoming a global problem for human health and the environment. Antibiotic resistance is exacerbated by the ability of bacteria to share their antibiotic resistance genes (ARGs) with other bacteria via horizontal gene transfer (HGT). Many existing studies on HGT of ARGs focused on antibiotic concentrations at or above the minimal inhibitory concentration (MIC), which is the lowest concentration of an antibiotic that prevents visible growth of a bacteria culture. However, knowledge on the development of antibiotic resistance under different stressors at sub-MIC levels is still limited. In addition, carbon nanotubes (CNTs) have been widely studied in environmental, agricultural and biomedical areas due to their unique physical and chemical characteristics, but limited studies have been done to evaluate the effects of CNTs on the spread of ARGs. Electrochemical filtration has been shown to be a cost-effective technique to remove recalcitrant compounds and reduce antibiotic resistance, but limited studies have been done to evaluate the effectiveness of removal of ARGs with electrochemical filtration. Therefore, there is a critical need to evaluate the effects of trace levels of antibiotics and CNTs on the development of antibiotic resistance and electrochemical removal of ARGs.

The specific research objectives of this study were to evaluate: (1) selective pressure of sub-inhibitory concentrations of antibiotics on the development of antibiotic resistance and HGT, (2)

development of antibiotic resistance and HGT under exposure to CNTs and antibiotics, and (3) effectiveness of using an electrochemical MWCNT filter to remove ARGs.

To evaluate the development of antibiotic resistance exposed to sub-MIC of erythromycin, HGT between environmental donor (*E. coli*) and pathogenic bacterial recipient (*B. cereus*) was quantified. The results indicated that extremely low concentration (0.4 ng/L to 4 μg/L) of erythromycin promoted HGT of *erm*80 gene, which is an erythromycin resistance gene. In addition to traditional culture-based method and quantitative real-time PCR (qPCR), a fluorescence *in situ* hybridization (FISH) approach was used to detect the occurrence and development of ARGs even the bacteria were in the viable but nonculturable (VBNC) state after treatment of sub-lethal level of erythromycin. Multi-walled carbon nanotubes (MWCNT) was selected as a representative stressor to evaluate the effects on HGT. The results showed that MWCNT enhanced HGT above 1 × MIC, which is the lethal level of erythromycin to recipients, and transfer frequencies of *erm*80 genes increased up to 101-fold under exposure to 1 × MIC erythromycin and MWCNT as compared to no MWCNT control. However, transfer efficiency of *erm*80 gene under exposure to sub-MIC of erythromycin was inhibited by MWCNTs. Moreover, transfer of antibiotic resistance plasmids was affected by antibiotics and MWCNTs. Although the concentration of individual stressor was not enough to confer antibiotic selection, effects of both antibiotics above 1 × MIC and MWCNTs could add up and select for antibiotic resistance. The results suggested that CNTs might create additional selective pressure for the spread of ARGs and their effects on HGT should be further investigated. Finally, an electrochemical MWCNT filtration was evaluated to remove genomic DNA and ARGs under the effects of operating conditions, such as pH, phosphate, and NOM. The results showed that the electrochemical MWCNT filtration reactor achieved 79% removal efficiency for genomic DNA and 91% removal efficiency for *erm*80 genes. The study suggested that electrochemical MWCNT filtration could be a promising technology for the removal of DNA and ARGs.

Overall, the results improved our understanding of the development of antibiotic resistance and ARGs under various selective pressures. Trace levels of antibiotics promoted the development and spread of ARGs. Conjugative transfer of resistance genes exposed to sub-MIC levels of erythromycin and MWCNTs also contributed to the spread and propagation of ARGs. As antibiotic

concentrations detected in natural environment are often in trace levels, the results of this study may improve the understanding of health risks of trace levels of antibiotics and help develop effective mitigation strategies to control the spread of antibiotic resistance. Effective removal of ARGs with electrochemical MWCNT filtration may help the development of cost-effective treatment systems to remove ARGs to protect human health and the environment.

CHAPTER 1. INTRODUCTION

1.1 History of antibiotic and the development of antibiotic resistance

The definition of antibiotic was first proposed by Selman Waksman, who discovered streptomycin. At first, this definition just simply referred to any small molecules that antagonize the growth of microbes. Today, the term "antibiotic" is used to define an organic molecule that kills or inhibits the growth of another microorganism (Davies et al., 2010). The discovery of antibiotics is one of the most important health-related events of the contemporary era because it affects the treatment of infections and other therapeutic applications of antibiotics like anticancer or antitumor agent. A timeline of antibiotic discovery and antibiotic resistance development is shown in Figure 1-1.

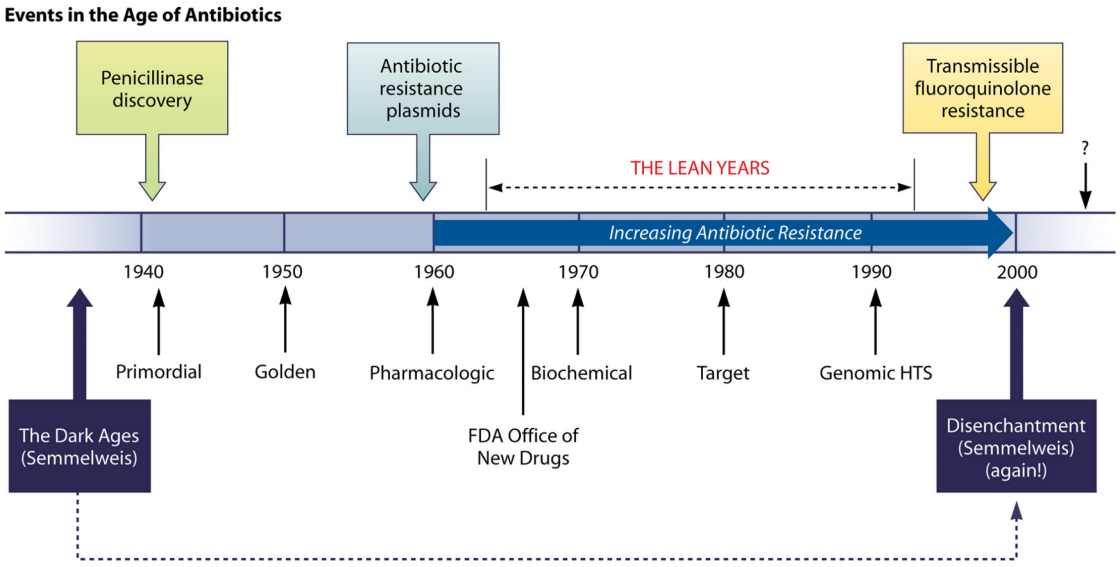

Figure 1-1 History of antibiotic and antibiotic resistance (Davies & Davies, 2010). The dark ages are the years before discovering antibiotics; primordial means the beginning of chemotherapy; people found the most of antibiotics we are using today in the golden era; pharmacologic period improves understanding on the usage of antibiotics on patients; during biochemical era, studies on biochemical actions and mechanisms of antibiotic resistance were made to avoid antibiotic resistance; in the 1980s marked as target, new antibiotic compounds were designed via genetic methods; in the decade of genomic HTS (high-throughput screening), people used genome sequencing to find important targets to incorporate into HTS assays; disenchantment marked the time when companies stopped their discovery of new antibiotics due to the failure of applying genome-based methods. During the lean years, the discovery and development of new antibiotics were reduced. FDA (Food and Drug Administration) established strict requirements to control the use of antibiotics for drug safety, and Dr. Semmelweis promoted hand disinfection as an antiseptic procedure before discovering antibiotics to prevent infections.

The modern era of antibiotics started with the discovery of penicillin from *Penicillium notatum* by Alexander Fleming in 1928. Before using penicillin as a therapeutic agent, penicillinase which belongs to β-lactamase, was identified by Abraham and Chain in 1940 (Abraham et al., 1940). In 1944, streptomycin was introduced to treat tuberculosis (TB), and a large number of *Mycobacterium tuberculosis* resistant bacteria were found during patient treatment. Similar results have ensued after many other antibiotics have been discovered and used in therapeutic applications. During the golden era, most of the antibiotics that we are using today were discovered. The use of antibiotics for non-human applications, such as animal growth promotion, began in the early 1950s and expanded enormously (Davies & Davies, 2010). Unfortunately, antibiotic resistance also rapidly developed with the discovery of antibiotics.

Antibiotic resistance is the acquired ability of a microorganism to grow in the presence of an antibiotic to which the microorganism is usually sensitive (Black, 2012). In the mid-1950s, Japanese biologists S. Mitsuhashi, R. Nakaya, and T. Watanabe revealed that antibiotic resistance gene could be transferred by bacterial conjugation, which is a horizontal gene transfer (HGT) process (Davies, 1995). Antibiotic resistance transfer by plasmids has been considered as a major route for antibiotic resistance development because of its practical significance (Norman et al., 2009). Nowadays, more studies have focused on the mechanisms of antibiotics and antibiotic resistance development.

1.2 Health risks of antibiotics

Antibiotics are manufactured and used throughout the world, and their widespread use has resulted in a major health concern. In 2001, more than 10,000 metric tons of antibiotics were used for non-therapeutic purposes as growth promotion and prophylactics in aquaculture in the United States (Kümmerer, 2009). Among the large amount of antibiotics produced, about 75% of antibiotics cannot be absorbed by animals and are excreted into the environment (Chee-Sanford et al., 2009). Therefore, antibiotics could transfer to and contaminate the environment and lead to increased levels of antibiotic resistant bacteria and resistant genes. As a result, the morbidity and mortality of infections would raise and antibiotic resistance is becoming an emerging threat to public and environmental health (Colomer-Lluch et al., 2011). In addition, the increasing use of antibiotics

for both therapeutic and prophylactic applications have resulted in the selection of bacterial pathogens resistant to multiple drugs. Multidrug resistant (MDR) organisms, which are also called superbugs, make the therapy more dangerous, more costly and sometimes more prone to failure.

1.3 HGT and stressors for selection of ARGs

The emergence and spread of ARGs among pathogenic bacteria are becoming a severe public health concern in recent years. It is increasingly recognized that pathogenic bacteria can acquire ARGs via HGT in environments. HGT is an exchange process of genetic information between organisms, other than the usual (vertical) inheritance process from mother cells to daughter cells (Furuya et al., 2006). HGT can facilitate the dissemination of ARGs from commensal and environmental species to pathogens. Three identified mechanisms of HGT include conjugation, transformation, and transduction (Figure 1-2).

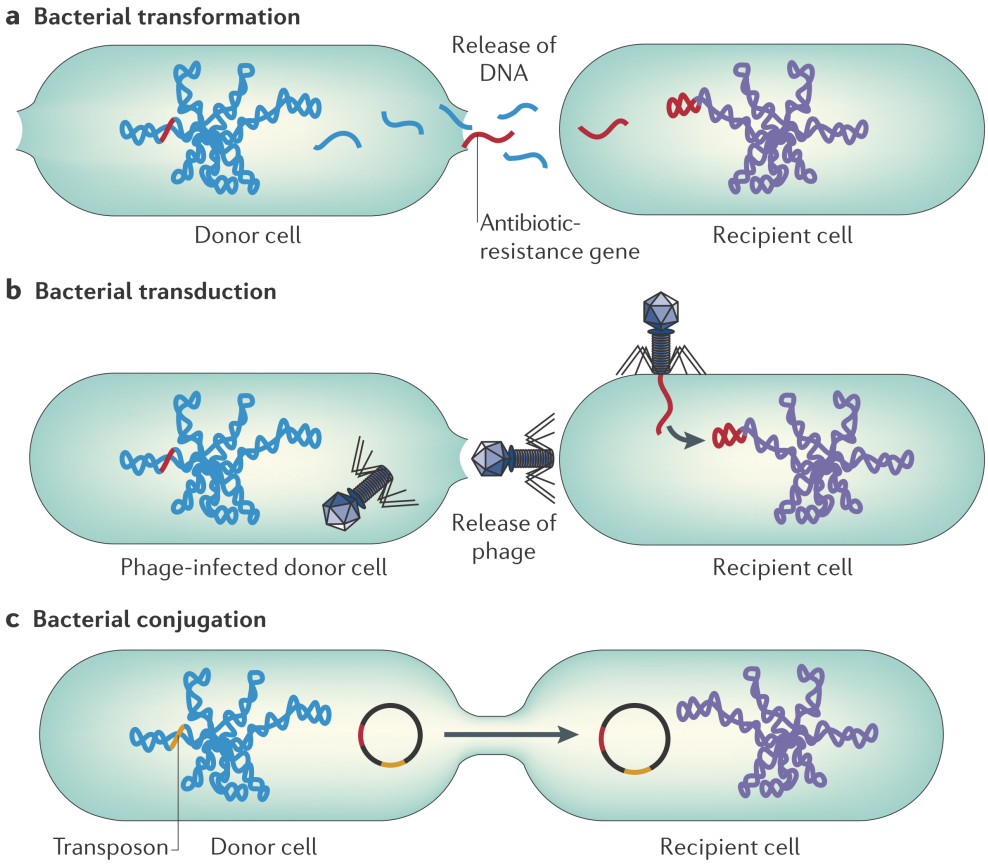

Figure 1-2 Horizontal gene transfer between bacteria (Furuya & Lowy, 2006). There are three mechanisms in HGT. (A) transformation. (B) transduction. (C) conjugation.

Transformation transfers genetic information via naked DNA. Transduction involves transfer of host genes from one cell to another by a bacteriophage that acts as a vector for its injection into the recipient cell. Conjugation transfers DNA requires a cell-to-cell contact. Among the three mechanisms, conjugation is considered to have the greatest influence on the dissemination of ARGs, because many resistance genes are placed on mobile genetic elements, such as plasmids and transposons. Transposons are sequences of DNA that carry their own recombination enzymes that allow for transposition from one location to another and transposons can also carry ARGs. Understanding how ARGs transferred among bacteria via HGT is essential to control the dissemination of antibiotic resistance.

Environmental selective pressure, such as antibiotics, metals, nutrients, and disinfection byproducts (DBPs), can affect ARGs acquisition rate by HGT. HGT could occur in natural environments under carbon-limited conditions that do not normally select for plasmids. Fernandez-Astorga et al. (1992) mixed an *E. coli* containing an ampicillin and gentamicin resistance plasmid with a plasmid-free *E. coli* under conditions of less than 1 mg/L TOC and they found that the number of transconjugants and transfer frequency were affected by the concentrations of TOC in mating experiments and the conjugal frequency under 1 mg/L TOC was still reached to 10^{-4} (Fernandez-Astorga et al., 1992). Previous studies showed that metal contamination functioned as a selective pressure for the dissemination of antibiotic resistance (Alonso et al., 2001; Summers et al., 1993). Unlike antibiotics, heavy metals cannot be readily degraded and exert a long-term selection pressure on environmental bacteria and contribute to selection of ARGs (Martinez, 2009). DBPs (chlorite and iodoacetic acid) are antibiotic-like chemicals and at both minimum inhibition concentration (MIC) and sub-MIC levels can select for antibiotic resistance (D. Li et al., 2016).

1.4 Erythromycin

Erythromycin is the first discovered macrolide antibiotic and has been used for effective treatment of infections caused by some intracellular pathogens, such as *Legionella* and *Chlamydia*, since the 1950s (Zuckerman, 2004). Moreover, it is applied in livestock and poultry production to promote animal growth, prevent diseases, and increase feed efficiency (Jessick et al., 2011). Erythromycin is a crystalline compound that is slightly soluble in water but soluble in most of common organic

solvents (Flynn et al., 1954). It has a moderate spectrum and is effective against Gram-positive and some Gram-negative bacteria (Marilyn C. Roberts, 2008) through inhibiting RNA-dependent protein synthesis by reversibly binding to the 50S ribosomal subunit of sensitive bacteria (Zuckerman, 2004). The inhibition of bacterial growth is resulted from dissociation of peptidyl-tRNA from the ribosome during the elongation phase (Omura, 2002).

1.5 Mechanisms of erythromycin resistance

Bacterial resistance to erythromycin could be divided into two ways: active or passive. Active resistance to erythromycin can be reached by three mechanisms: rRNA methylation, efflux-mediated resistance, and macrolide inactivation (Marilyn C. Roberts, 2008; Varaldo et al., 2009; Zerrin et al., 2007). Passive resistance is generated by the adaptive processes that are not essentially linked to a given class of antibiotics (G. Wright, 2005). *E. coli* is Gram-negative bacteria that could be intrinsically resistant to low concentrations of erythromycin due to the impermeability of cell membrane (Arthur et al., 1987; R. Leclercq et al., 1991).

The most common mechanism of macrolide resistance is through rRNA methylation, i.e., the adenine-N^6 methyltransferase generates posttranscriptional modification of 23S rRNA by adding one or two methyl groups to a single adenine in the 23S rRNA moiety or one of the adjacent residues in the peptidyl transferase region (Marilyn C. Roberts, 2008; Varaldo et al., 2009). These enzymes further inhibit binding of antibiotics to its ribosomal target and become erythromycin resistant via the modification (Zerrin et al., 2007). The genes responsible for these methylations are named as erythromycin ribosome methylation (*erm*) genes (Marilyn C. Roberts, 2008). These genes can confer resistance to macrolides and also contribute to cross-resistance among macrolides, lincosamides, and streptogramin B, which are identified as MLS_B phenotype (Zerrin et al., 2007). A previous study has reported more than 30 different types of *erm* genes which are detected from different Gram-negative and Gram-positive bacteria (Marilyn C. Roberts, 2008). Due to the different phenotypes' expressions, *erm* genes could be divided into two categories: inducible and constitutive. Inducible *erm* genes are generally regulated by translational attenuation of the mRNA leader sequence or rho factor-independent termination (Marilyn C. Roberts, 2008). Bacteria with inducible *erm* genes are resistant to inducers and remain sensitive to non-inducer MLS_B antibiotics (Zerrin et al., 2007). Constitutive *erm* genes are controlled by structural alterations in the *erm*

translational attenuator (Marilyn C. Roberts, 2008), and mRNA is active even in the absence of inducers and conferring the high level cross-resistance to MLS_B antibiotics (Roland. Leclercq, 2002)..

Efflux proteins are resistant to low levels of MLS_B antibiotics by pumping out the antibiotics of the bacterial cell and making the intracellular concentrations stay low and ribosome not binding with antibiotics (M. C. Roberts et al., 1999). Current study showed that 14 different genes are coded for efflux proteins (Marilyn C. Roberts, 2008), and *mef*-class genes and *msr*-class genes encoded active efflux proteins and ABC transporter superfamily, respectively (M. C. Roberts et al., 1999; Varaldo et al., 2009).

Macrolide inactivation is associate with the synthesis of modifying enzymes which could selectively target and terminate the activity of macrolide antibiotics (G. Wright, 2005). Three types of enzymes (esterase, phosphotransferase, and glycosyltransferases) are responsible for macrolide inactivation (G. Wright, 2005). Esterases are encoded by erythromycin esterification (*ere*) genes, and these *ere* genes were first detected in *E. coli* strains which conferred to high levels of resistance to erythromycin (MIC ≥ 1600 μg/ml) (G. Wright, 2005). Moreover, the *ere* genes are located in mobile genetic elements, which make them prevalent in microbial communities (G. Wright, 2005). A previous study has illustrated that the *ere* genes were detected in several Gram-positive and Gram-negative bacteria (Marilyn C. Roberts, 2008). Phosphotransferases (MPHs) is able to inhibit macrolides by phosphorylation to the free hydroxyl of the desosamine sugar of macrolides that react with the 23S rRNA and generate resistance (G. Wright, 2005). These enzymes are encoded by macrolide phosphotransferase (*mph*) genes, and they were also first detected in *E.coli* strains (M. C. Roberts et al., 1999). Bacteria with the *mph* genes could have very high MICs (> 2000 mg/ml) to macrolides (G. Wright, 2005). Glycosyltransferases is a self-protection mechanism which could catalyze glucosylation at the desosamine sugar of macrolides to confer resistance in antibiotic-producing organisms (G. Wright, 2005).

1.6 Effects of antibiotics on HGT

Bacteria have developed adaptive mechanims that either by rising their mutation rates or acquring ARGs by HGT in the presence of antibiotic pressure. Several studies showed that conjugation

induced SOS response as a mehcnisms for susceptible bacteria to survive in the presence of antibiotic and also positively influenced the development of antibiotic resistance, which involved a rise in the rates and frequencies of HGT (Baharoglu et al., 2010; Beaber et al., 2004; Prudhomme et al., 2003; Úbeda et al., 2005; X. Zhang et al., 2000). As reported from these studies, conjugation process induced SOS response and led to genetic changes that were associated with the mobilization of mobile genetic elements, such as integrating conjugative element with ARGs. SOS response can be triggered in response to DNA damage. For instance, *E. coli* O157:H7 exposed to fluoroquinolones induced gene expression of Shiga toxin by activating SOS response and enhancing the transfer of toxin gene to sensitive bacteria (X. Zhang et al., 2000). Another example was SOS induction by low dose of fluoroquinolone antibiotics resulted in replication and transfer of SaPlbov1 (a *Staphylococcus aureus* pathogenicity island) (Úbeda et al., 2005). Moreover, antibiotic-activated SOS response also results in the transmission of antibiotic resistance genes. SXT is a 100kb integrating conjugative element encoding resistance genes to chloramphenicol, sulphamethoxazole, trimethoprim, streptomycin and heavy metals in *Vibrio cholera*. SXT was transferred and integrated more efficiently when the *Vibrio cholera* was exposed to sub-MIC antibiotic concentrations. SetR is a repressor protein that regulates the ability of SXT transfer, and the induction of SOS response would alleviate this repression (Beaber et al., 2004). The induction of competence can increase the rate of DNA transformation in *S. pneumoniare* by sub-inhibitory concentrations of aminglycoside and fluoroquinolone (Prudhomme et al., 2003).

Sub-inhibitory concentrations of antibiotics can also increase the rates of homologous recombination. Fluoroquinolone at sub-MIC antibiotic concentration can stimulate intrachromosomal recombination between divergent sequences and conjugational recombination of antibiotic resistance genes. Both of these two stimulations are independent of SOS induction (Lopez et al., 2007). Antibiotics that induce SOS responses can also enhance the rates of integrin recombination in *V. cholera* and *E.coli* (Guerin et al., 2009). Genes conferring resistance to many different classes of antibiotics and to disinfectants have been found in the form of particular ‘genes cassettes’ that collectively form an important gene pool. These cassettes can exist transiently in a free circular form (Collis & Hall, 1992) but do not include all of the functions necessary for their own movement and are usually associated with gene capture and expression elements called integrons (Stokes & Hall, 1989).

Most of our current understandings of selection of antibiotic resistance are at concentration above MIC, but the sub-inhibitory concentration of antibiotics is an important contributing factor for antibiotic resistance evolution. The sub-MIC of antibiotics can influence HGT and induce *intI*1, which is regulated by SOS response in bacteria (Guerin et al., 2009). However, our knowledge of antibiotic resistance transfer under the weak selective pressure is still limited.

1.7 Treatment technologies to remove ARGs

Several treatment technologies have been used to remove ARGs, which include photocatalytical processes (Dunlop et al., 2015; Rizzo et al., 2014; Xiong et al., 2013), ozone oxidation (Oh et al., 2014), UV disinfection (Guo et al., 2015; Pang et al., 2015), and chlorination (Zhuang et al., 2015). However, most existing treatment technologies are either expensive to operate or causing further pollutions which need follow-up treatments. In recent years, CNTs have gained increasing attention for environmental applications in water treatment due to their high electrical conductivity, high specific surface area, and high porosity (Das et al., 2014; Q. Li et al., 2008; Mauter et al., 2008). SWCNT and MWCNT filters were found to be effective to remove *E. coli*, virus, antibiotic tetracycline and natural organic matter (NOM) (Brady-Estévez et al., 2008; Hyung et al., 2007; Y. Liu et al., 2015; Vecitis et al., 2011). Furthermore, MWCNT filters were reported to have a higher removal efficiency of viruses than SWCNT filters due to a more uniform CNT-matrix on the MWCNT filters (Brady-Estévez et al., 2010). However, no studies have been done to remove ARGs using electrochemical MWCNT filters. To minimize potential health risks posed by ARGs, it is important to examine the feasibility of using MWCNT membrane to remove ARGs.

1.8 Objectives

The widespread use of antibiotics has driven selection for resistance in bacteria, which is becoming a serious problem for human health and environment. Several factors, such as antibiotics, may promote the development of antibiotic resistance in natural microbial communities and ARGs could transfer from non-pathogens to pathogens. Most existing studies focused on selection and spread of ARGs under high levels of selective pressures. However, the knowledge on the development of antibiotic resistance under different stressors at sub-MIC levels is still limited.

Therefore, it is pivotal to study the ability of low levels of stressors to select for bacterial resistance. Cost-effective treatment technologies to remove ARGs are also limited.

The overall objective of this study is to evaluate the development of antibiotic resistance under exposure to sub-MIC of antibiotics and MWCNT and evaluate removal efficiency of electrochemical filtration of ARGs.

The specific research objectives were to:

I) evaluate selective pressure of sub-inhibitory concentrations of antibiotics on the development of antibiotic resistance and HGT;

II) evaluate the development of antibiotic resistance and HGT under antibiotics and carbon nanotubes;

III) evaluate the potential of an electrochemical MWCNT filter for the removal of bacterial DNA and ARGs.

CHAPTER 2. EFFECTS OF TRACE LEVEL ANTIBIOTICS ON THE DEVELOPMENT OF ANTIBIOTIC RESISTANCE AND HORIZONTAL GENE TRANSFER

2.1 Introduction

2.1.1 The sub-MIC environment

Previous studies showed that only antibiotics with low concentrations in the ng/L to μg/L range can be naturally produced by bacteria and fungi (Kümmerer, 2009), while commercial applications introduce larger amounts of antibiotics of high concentrations into the environment. The typical uses of antiobics include: (I) therapeutic and preventive purposes in humans; (II) growth promotion, therapeutic, and preventive uses in aquaculture; (III) promotion of growth, feed efficiency and preventive uses in animal husbandry; (IV) pest control and preventive use in agriculture; (V) usage as biocides in household cleaning. Resistant bacteria and antibiotics can disseminate between different environments, such as medical environment, aquacultural environment, agricultural environment, natural environment, and pharmaceutical industry, and create a range of selective presssures for the development of antibiotic resistance in bacteira.

Antibiotics exert their effects under different active concentrations in various environments and ecosystems. Antibiotics used in humans, animals, agriculture, and aquaculture are generally present at high concentrations and exert strong selective pressures on bacteria. A large percent of these antibiotics that are used therapeutically and prophylactically in humans and animals are further released into the natural environment in an unchanged active form, via urine, feces and intentional release of drugs. These antibiotics will end up as diluted antibiotics in lakes, rivers and soils. Contrary to traditional thoughts that the antibiotic concentration has to exceed MIC to post a selective pressure, antibiotics with low concentrations in the natural environments exert weak selective pressures on bacteria and contribute to the selection of ARGs.

2.1.2 Selection dynamics at sub-inhibitory antibiotic concentrations

Traditionally, it has been assumed that resistant bacteria could be selected at antibiotic concentrations between the MIC of the susceptible wild-type strain (MIC_{susc}) and the MIC of the resistant strain (MIC_{res}). The antibiotic resistance field (Drlica et al., 2007) was dominated by the

mutant selective window hypothesis that the antibiotic concentrations lower than the MIC_{susc} did not inhibit the growth of bacteria and were not selective. The sub-MIC selective window covered a wider order of magnitude than the traditional selective window (Figure 2-1).

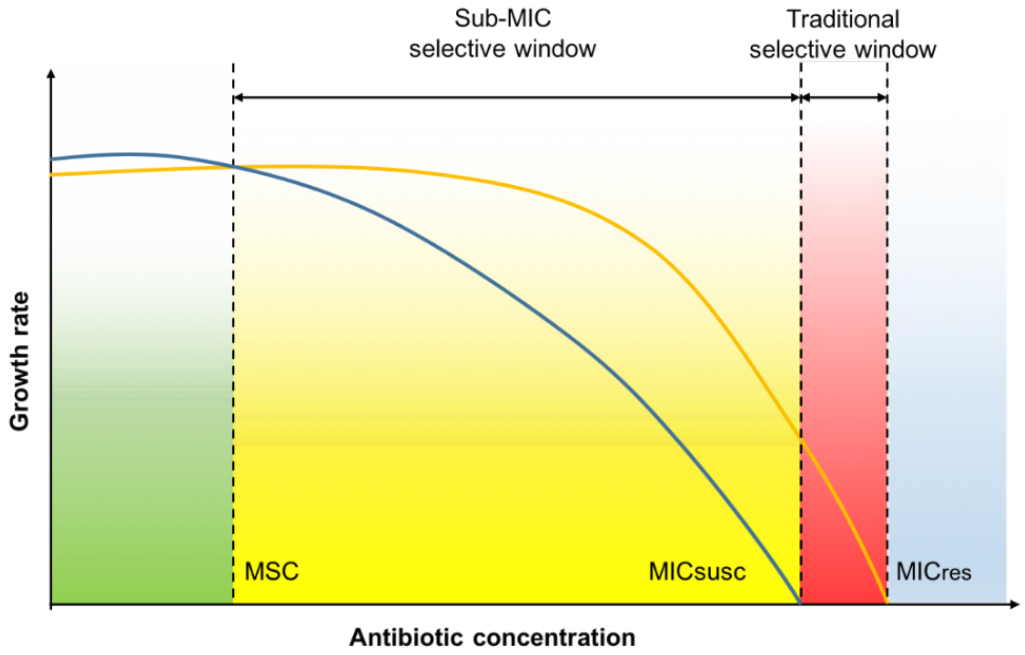

Figure 2-1 Sub-MIC selective window (Andersson et al., 2014). Green region with lowest concentration represents when the susceptible strain (blue line) outcompetes the resistant strain (orange line), while yellow and red concentration intervals indicate otherwise. Yellow region indicates the sub-MIC concentrations of antibiotics and red interval indicates high concentrations of antibiotic.

Contrary to the traditional view, two recent studies showed that the mutants could also be selected between the concentration of minimal selective concentration (MSC) and MIC_{susc}, as the sub-MIC selective window (Gullberg et al., 2011; A. Liu et al., 2011). Gullbert et al. used a flow cytometry method to determine the MSC by growth competitions between isogenic resistant and susceptible strains that differentially carrying fluorescent proteins (cyan fluorescent protein, CFP and yellow fluorescent protein, YFP) and found that antibiotic resistance cells can be selected at the sub-MIC concentration, which was several hundred-fold below the MIC (Gullberg et al., 2011). A ciprofloxacin-resistant mutant (*gyrA S83L*) can be selected at a concentration as low as 0.1 ng/ml (the MSC of this mutant), which was 1/230 of the wild-type MIC level. The sub-MIC selective window for this mutant spanned a wider order of magnitude (from 0.1 ng/ml to 23 ng/ml) than the

traditional selective window (from 23 ng/ml to 380 ng/ml) (Gullberg et al., 2011). In another study, Liu et al. used chromogenic proteins as color markers to determine the MSCs for ciprofloxacin, which was 1/5 of the MIC, and tetracycline, which was 1/20 of the MIC, of resistance mutations in *E. coli* (A. Liu et al., 2011).

Although several studies have illustrated positive correlations between low level antibiotics and selection of ARGs (Gullberg et al., 2014; Gullberg et al., 2011), studies on the effects of sub-MIC antibiotics on the development of antibiotic resistance are still limited.

2.1.3 Evaluation of HGT of ARGs

To date, three main approaches have been widely used to investigate the horizontal transfer of ARGs in natural environments: culture-based, molecular-based, and fluorescence-based methods, each of which has its own advantages and limitations. Both culture-based methods and molecular microbiology techniques have been used to evaluate HGT of ARGs. Traditionally, bacteria in natural environments are detected and quantified by culture-base methods. Many studies have applied culture-based methods to investigate the occurrence and dissemination of antibiotic resistance bacteria and ARGs in aquatic systems, but usually only less than 1% of bacteria in total microbial community can be cultivated (Yim et al., 2007). At the end of the growth curve, some of the culturable cells enter viable but nonculturable (VBNC) which fail to form colonies due to environmental stress while the total cell counts remain stable. Unfortunately, VBNC cells may be a health concern if they are pathogenic and can cause infections in human and animals (Trevors, 2011). VBNC bacteria retain their virulence for a certain period of time and can become culturable again under favorable environmental conditions (J. D. Oliver, 2010). Although VBNC cells present low metabolic activities, they could be resistant to antibiotics (J. D. Oliver, 2010). VBNC cells with ARGs may have significant clinical implications as they have the potential to spread ARGs but conventional culture-based methods are not effective to detect them. Therefore, it is important to monitor VBNC cells in environments and molecular microbiology tools are needed to evaluate antibiotic resistance in total microbial community.

Many studies have shown that qPCR is an effective approach to examine the occurrence and abundance of different ARGs in natural environments (M. Liu et al., 2012; Mao et al., 2015; Oberle

et al., 2012), but it may not accurately estimate the abundance of ARGs as it is largely dependent on available primers and ARG databases. The advantages of real-time qPCR method over traditional plate-counting method are that it is quantitative, culture-independent, and has high sensitivity (down to one copy of gene). However, there are some drawbacks for using real-time PCR approach that may impact our results. The most important limitation is that it is unable to distinguish the targeted genes in living bacteria from those in dead bacteria. Besides, it cannot discover whether the *erm*80 genes are in pathogens or in non-pathogens. More importantly, qPCR method is incapable of differentiating among donor cells with multiple ARGs, antibiotic-sensitive recipients, and transconjugants in the mating experiments. The real-time PCR method could not provide any information about gene expression of ARGs. Additional techniques are needed to fill in the gap.

Fluorescence *in situ* hybridization (FISH) is an approach to detect specific rRNA sequences *in situ* by designed fluorescently labeled probes (Amann et al., 1990). A FISH technique was developed to quantify erythromycin resistance due to rRNA methylation of 23S rRNA, which is resulted from express *erm* genes (Zhou et al., 2009). Combining with phylogenetic probes, this technique can quantify antibiotic resistance in specific phylogenetic groups in total microbial community, which include both culturable microorganisms and VBNC cells (Zhou et al., 2009).

The objective of this study was to evaluate the effect of sub-MIC levels of antibiotics on horizontal transfer of ARGs. Plate count method, qPCR, and FISH were used to comprehensively evaluate transfer of *erm* genes and mobile genetic elements through conjugation. The study can help assess HGT of ARGs across different groups of bacteria and improve environmental risk assessment of trace levels of antibiotics in the environment.

2.2 Materials and Methods

2.2.1 Type and growth of bacteria

To study HGT of *erm* genes in natural environment, an erythromycin-resistant donor and erythromycin-sensitive recipient were isolated from the natural environment. An *Escherichia coli* (*E. coli*) strain was selected as the donor as it harbors plasmid pTE80, which confers antibiotic

resistance to erythromycin. *erm*80 gene was detected in pTE80 plasmid after screening. On the other hand, we selected Gram-positive *Bacillus cereus* (*B. cereus*) as erythromycin sensitive recipient strains in this study. *B. cereus* is a human pathogen that is commonly distributed in natural environments and is mostly associated with food poisoning. The Gram-positive/Gram-negative pair was chosen to evaluate if HGT occurred between significantly different species of bacteria.

The *E. coli* with plasmid pTE80 and *B. cereus* were originally isolated from Wabash River, West Lafayette, IN, and were further identified by sanger sequencing. After using 16S rRNA primers (533F and 1492R) to run PCR amplification, purified PCR samples were sent to Purdue genomic core facility for sequencing. The donor strain was identified as *E. coli* and recipient strain was identified as *Bacillus cereus* (ATCC 14579) through analysis with BLASTn, which is a nucleotide basic local alignment tool developed by National Center for Biotechnology Information (NCBI). The alignment results of ATCC 14579 (complete genome) and pTE80 showed no significant similarity between donor and recipient strains.

2.2.2 Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) for *E. coli* and *B. cereus* were determined as previously described (Wiegand et al., 2008). To obtain a single colony for MIC analysis, bacterial suspensions were streaked onto lysogeny broth (LB) agar plates without any inhibitors. A colony was then isolated from the agar plate and inoculated to LB media for incubation until the broth reached a visible turbidity that was equal to the turbidity of a 0.5 McFarland Standard (1.175% (wt/vol) $BaCl_2 \cdot 2H_2O$ and 1% (wt/vol) H_2SO_4), which indicates a concentration of 1×10^8 colony forming units (CFU)/mL. Donor and recipient bacteria were added into test tubes containing LB broth and supplemented with 0, 0.1, 0.2, 0.25, 0.3, 0.35, 0.4, 0.45, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 2, 4, 8, 16, 32, 64, and 128 mg/L of erythromycin and incubated overnight at 37 ºC in an incubator shaker at 180 rpm. The MIC was identified as the lowest concentration of an antibiotic that completely inhibited the visible growth of a microorganism. The determined MICs of erythromycin for *E. coli* and *B. cereus* were 64 mg/L and 0.4 mg/L, respectively.

2.2.3 Bacterial viability test

Different concentrations of erythromycin (Table 2-1) were added to mixture of donor and recipient bacterial suspensions. After incubating at 37 °C for 4 hours, 100 μL of bacterial suspension of each sample before and after the treatment was serially diluted and spread on LB agar plates. LB agar plates were incubated at 37 °C overnight. The viable cells were enumerated by counting CFUs on LB agar plates. All experiments were performed in triplicate. Viability rate of bacterial cells was calculated as the ratio of the number of viable cells under erythromycin treatment and the number of viable cells without erythromycin treatment.

Table 2-1 Concentrations of erythromycin used in mating experiments

Concentrations (mg/L)	0	4×10^{-7}	4×10^{-5}	4×10^{-3}	0.4	4
Corresponding MIC	0	10^{-6}	10^{-4}	10^{-2}	10^{0}	10^{1}

2.2.4 Mating experiments

Mating experiments were performed using *E. coli* as donor strain and *B. cereus* as recipient strain. The experiments were conducted in two conditions, with gradually increasing erythromycin concentrations up to 10 × MIC of recipient strain (referred as high concentration selection) and lower concentrations of erythromycin (sub-MIC). The experiments were conducted under different concentrations of erythromycin ranging from 4×10^{-7} mg/L to 4 mg/L. The initial ratio of donor and recipient cells was 1:10, and transconjugants were selected on erythromycin (0.4 mg/L) and polymyxin B (100000 IU/L) LB selective medium. The donor and recipient cultures were grown separately in LB medium in a shaking (140rpm) incubator at 37 ºC until they reached log phase. The donor cells were washed twice in 1 × PBS buffer to remove traces amount of erythromycin before the mating experiments. In mating experiments (Figure 2-2), donor strains (10^5 CFU/mL) and recipient strains (10^6 CFU/mL) were mixed and incubated in LB broth supplemented with different concentrations of erythromycin (Table 2-1).

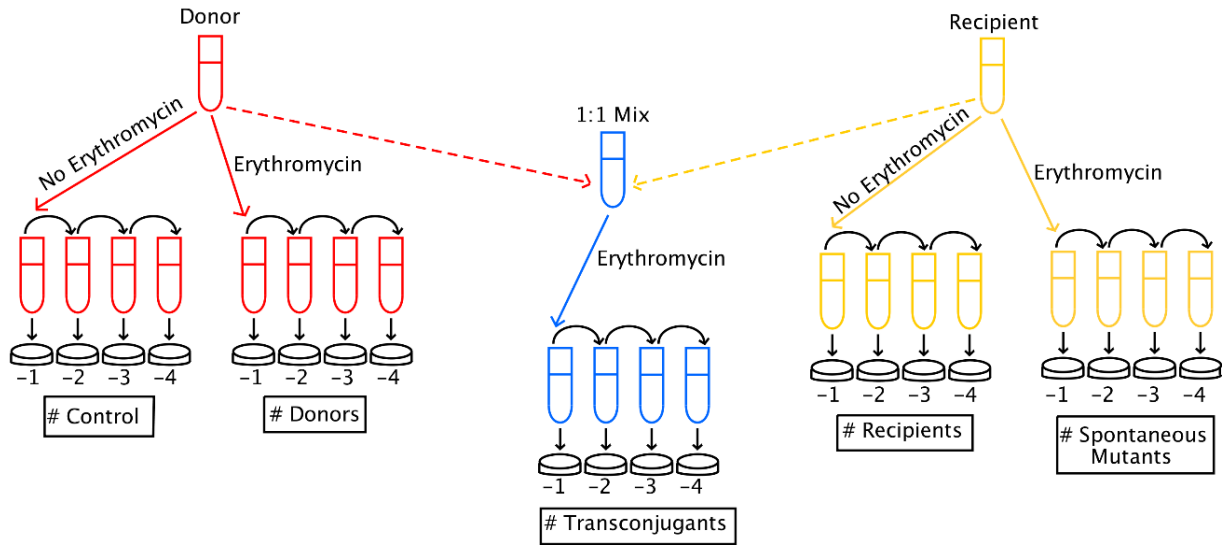

Figure 2-2 Schematic diagram of the mating experiment. Donors with *erm*80 plasmid (red), recipients (yellow) and a mixture of them (1:1 volume, blue) separately grown on LB agar with different concentrations of erythromycin. Plate counting method was applied to measure transconjugants. Tenfold serial dilution plating was used to estimate the CFU of bacteria in this study. (-1=10^{-1}, -2=10^{-2}, -3=10^{-3}, -4=10^{-4}).

Samples were collected after incubation for 0, 12, 24, 36, 48 hours and used for plate counting. Serial dilutions in 1 × PBS buffer were used and aliquots (100 µL) were plated onto LB-selective agar containing polymyxin B and erythromycin and LB agar plates in triplicate. All plates were incubated at 37 ºC. Transconjugants selected with selective LB plates, and recipients and donors were counted by plating on LB agar plats without erythromycin and polymyxin B. To check for the occurrence of spontaneous mutations, only recipient bacteria were incubated similar conditions and plated on selective medium.

Horizontal transfer frequency was calculated using the following equation:

$$\text{Transfer frequency} = \frac{\text{transconjugants (CFU/mL)} - \text{spontaneous mutants (CFU/mL)}}{\text{recipients (CFU/mL)}}$$

2.2.5 Identification of transconjugants with PCR

To confirm the presence of *erm*80 genes in transconjugants, one out of five colonies grown onto the selective media was analyzed by colony PCR. A fast boiling procedure was used to extract DNA for colony PCR. Briefly, freshly grown colonies were picked and dissolved in 50 µL TE buffer. The tubes were heated at 100 ºC for 5 min and spinned down for 30 sec, and finally 2 µL

solution was used as DNA template for PCR. In this study, oligonucleotide primers *erm*80 (Table 2-2) were designed to amplify a region of erythromycin resistance genes related to plasmid transfer (Genebank AF080450.1).

Table 2-2 PCR and qPCR primers and conditions.

Name	Target	Sequence (3'-5')	Annealing Temp (ºC)	Amplicon (bp)	References
533F	All bacteria	GTGCCAGCAGCCGCGGTAA	50	959	(Weisburg et al., 1991)
1492R		GGTTACCTTGTTACGACTT			(Turner et al., 1999)
Eub338F	All bacteria	ACTCCTACGGGAGGCAGCAG	60	180	(Muyzer et al., 1993)
Eub518R		ATTACCGCGGCTGCTGG			
*erm*80F	*erm*80 resistant gene	ACTTACCCGCCATACCACAG	60	233	this study
*erm*80R		AGGGAGTTGTTTCCTCCCGT			
*qIntI*1F	Class 1 integrase gene	ACCAACCGAACAGGCTTATG	60	286	(M. S. Wright et al., 2008)
*qIntI*1R		GAGGATGCGAACCACTTCCAT			

PCR amplification of DNA from individually picked colonies was conducted in a 50 µl reaction mix containing 14.25 µL GoTaq Flexi DNA polymerase (Promega, Madison, WI), 31.75 µL sterile nanopure water, 2 µL primers, and 2 µL DNA template. All reactions were performed in aC1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA) with the following thermal conditions: an initial denaturation at 95 ºC for 3 min, followed by 40 cycles of 30 sec at 95 ºC, 30 sec at corresponding annealing temperature (Table 2-2), and 30 sec at 72 ºC. Aliquots (10 µL) of the PCR products were mixed with 6 × DNA loading dye and analyzed using 1.5% (w/v) agarose gel electrophoresis containing SYBR Safe DNA gel stain (Life Technologies Ltd., Carlsbad, CA).

2.2.6 Quantification of *erm*80, *intI*1, and 16S rRNA with qPCR

To evaluate if integrons could facilitate HGT under exposure to sub-MIC levels of antibiotics, qPCR was applied to quantify class 1 integron (*intI*1) genes and *erm* genes. Samples from mixed culture (2 mL) were centrifuged and the total DNA was extracted as previously described (Russell et al., 2000). DNA extract yields and purities were quantified with a NanoDrop 2000c UV-Vis Spectrophotometer (Fisher Scientific, Hampton, NH). qPCR assays were also used to target the 16S rRNA gene for all bacteria. To estimate the potential contribution of class 1 integrons to the

dissemination of ARGs in natural environment, the comparisons between relative abundance of *erm*80 genes and *intI*1 genes were performed under different concentrations of erythromycin. All data were normalized by the gene copy numbers of 16S rRNA to calculate relative gene abundance. The details of the primers and amplification sizes are listed in Table 2-2.

Calibration standards for qPCR of each targeted gene were prepared from serial dilutions of purified PCR products. Each targeted gene was amplified from a positive control by conventional PCR. The amplicon size and specificity of PCR products were identified by comparison with 50 bp to 1000 bp DNA ladder after 1.5% (w/v) agarose gel electrophoresis. PCR products were then purified by the Wizard SV Gel and PCR clean-up kit (Promega, Madison, WI) according to the manufacturer's instruction. The concentration and purity of purified amplicon were examined using the NanoDrop 2000c and the absolute gene copy numbers were calculated by the known molar weight of each amplicon. At least four 10-fold dilutions of known gene copy numbers were generated as standards in each qPCR run.

The total volume of each reaction was 10 μL, which contained 5 μL 2 × iTaq universal SYBR Green supermix (Bio-rad, Hercules, CA), 0.2 μL individual primer (10 μM), 2 μl of DNA templates, and 2.6 μL sterile nanopure water. All reactions were carried out on a CFX96 Real-time PCR detection system (Bio-rad, Hercules, CA) with the following thermal conditions: an initial denaturation at 95 ºC for 3 min, followed by 40 cycles of 30 sec at 95 ºC, 30 sec of annealing at 60 ºC, and 30 sec at 72 ºC. Subsequently, melt curve analysis was performed by the increasing temperature from 65 ºC to 95 ºC. DNA templates, negative control (DNA template replaced by sterile nanopure water), and 10-fold serial dilutions of standard were analyzed in triplicate per qPCR experiment. All qPCR products were examined on 1.5% (w/v) agarose gels to confirm the presence of a single band at the appropriate amplicon size.

2.2.7 FISH Experiments

Real-time qPCR approach is better than traditional culture-based method because it is quantitative, culture-independent, and has high sensitivity, but it cannot provide information about *erm* gene expression. Therefore, dual-labeling FISH was conducted to detect methylation of 23S rRNA resulted from *erm* gene expression in recipient *B. cereus* strain. Two fluorescently labeled probes,

LGC354B probe and MLS_B probe, were used to target total numbers of *B. cereus* cells and the numbers of unmethylated MLS_B sensitive cells, respectively. The details of these two probes are listed in Table 2-3.

Table 2-3 Oligonucleotide probes used for FISH analysis.

Probe Name	Label (5')	Target Organism	Target Molecule	Formamide (%)[a]	Specificity (%)[b]	Coverage (%)[b]	Probe Sequence (5'-3')	Ref
MLS_B	Cy3	MLS_B sensitive bacteria	23S rRNA	12.5	100	82.8	GGGTCTT TCCGTC	(Zhou et al., 2009)
LGC354B	FAM	Firmicutes	16S rRNA	20	99.9	61.3	CGGAAG ATTCCCT ACTGC	(Meier et al., 1999)

a Formamide % is the percentage of formamide used in the hybridization buffer.
b Specificity and coverage of oligonucleotide probes were examined by SILVA ribosomal RNA databases (https://www.arb-silva.de/).

MLS_B resistance was indirectly quantified from the difference between the MLS_B sensitive cells and the total cells. MLS_B-sensitive *Enterococcus faecalis* JH2-2 (positive control) and MLS_B-resistant *Rhodococcus coprophilus* (negative control) that had one mismatch in the targeting methylation site were previously used to validate the MLS_B probe (Zhou et al., 2009). 0.3 mL of mating samples was collected and fixed with absolute ethanol for 2 hours in ice. The fixed samples were filtered through 0.22 μm polycarbonate membranes (Merck Millipore, Burlington, MA). Then the cells were transferred from the membrane to gelatin-coated slides, and washed for 3 min each in 50%, 80% and 95% ethanol for dehydration. Subsequently, 15 μL of hybridization buffer was mixed with 1 μL fluorescently labeled probes (50 ng/μL) and applied to each well. The hybridization buffer of MLS_B probe and LGC354B probe consisted of 1.8 M NaCl, 1 M Tris (pH 7.2), 10% sodium dodecyl sulfate (SDS), and formamide. The compositions of washing buffer were 1.8 M NaCl, 1 M Tris (pH 8), and 10% SDS. For dual-label FISH, samples were incubated with LGC354B probe first at 46 ºC for 2 hours in a moisture chamber under dark, and then with MLS_B probe for 2 hours at 37 ºC. After hybridization, all slides were rinsed with preheated washing buffer and washed for 20 min at 48 ºC and 37 ºC, respectively. The slides were stained by 1 μg/mL DAPI solution for 5 min, washed by sterilized nanopore water twice, and air dried. To prevent the photobleaching of fluorescence dyes, 5 μL Citifluor antifade mounting medium (EMS, Hatfield,

PA) was added to each well. Negative controls were performed with the same sample and hybridization buffer without probes and its average fluorescence intensities were employed as thresholds to classify positive and negative clusters by a modified fuzzy c-means clustering method as previously described (Zhou et al., 2007). All experiments were carried out in triplicate.

FISH images were captured under a Nikon Eclipse Ni-U Microscope equipped with DAPI, FITC and Cy3 filter cubes and a monochrome digital camera (Melville, NY). Between 5 and 15 locations, each of which contained between 500 and 800 cells, in each well were used to capture FISH images. The exposure times of DAPI, FAM, and Cy3 images were 0.5 s, 1 s, and 0.5 s, respectively. These images were automatically analyzed using Visilog image analysis software to detect individual cells stained with DAPI (excitation wavelength, 364 nm; emission wavelength, 454 nm), *Bacillus cereus* targeted by FAM-labeled probes (excitation wavelength, 495 nm; emission wavelength, 520 nm), and erythromycin resistant bacteria targeted by Cy3-labeled probes (excitation wavelength, 550 nm; emission wavelength, 564 nm) according to the manufacturer's introduction from Integrated DNA Technologies. The image size was 4,908 × 3,264 pixels (1 pixel = 0.07 μm). Detailed analysis and calculations were described previously (Zhou et al., 2007).

2.3 Results and Discussion

2.3.1 Phenotype of donor and recipient strains.

B. cereus formed white crenated colonies with about 5 mm in diameter on the selective medium, while the growth of *E. coli* was completely inhibited because of polymyxin B used in the selective medium (Figure 2-3). Polymyxin B was first discovered from *Bacillus polymyxa*. The polymyxin B sulfate salt can disrupt the permeability of the cytoplasmic membrane and is generally used for the treatment of Gram-negative bacterial infections. The result showed that addition of polymyxin B sulphate at a final concentration of 100 units per mL to the medium was sufficient to differentiate *B. cereus* from a mix culture of *E. coli* and *B. cereus*.

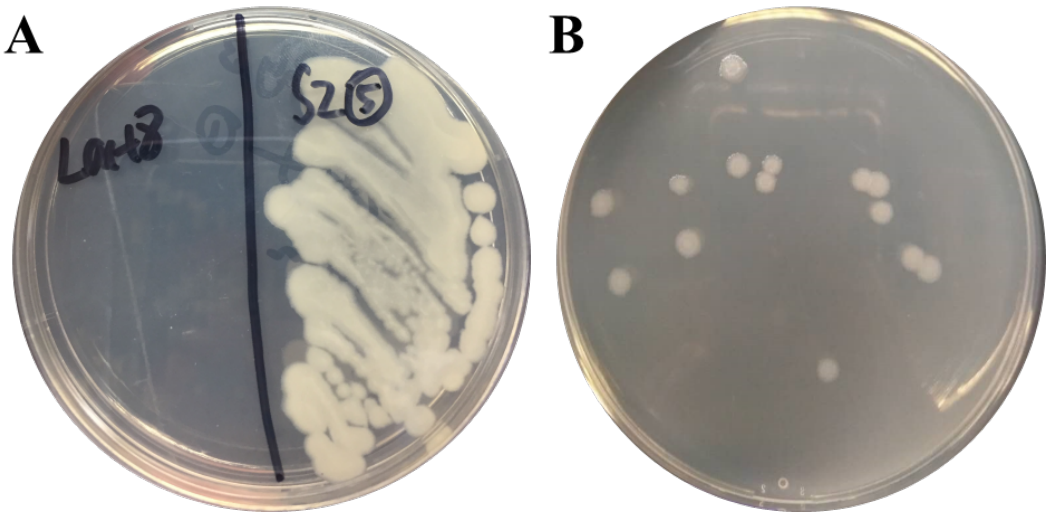

Figure 2-3 Selective medium to differentiate donor and recipient strains. (A) Colonies of *B. cereus* grown on selective medium were white (right side of the plate), but the *E. coli* were completely inhibited (left side). (B) Morphology of *B. cereus* colonies.

E. coli has been extensively studied and is one of the most commonly used prokaryotic model organisms in microbiology. *E. coli* is normally found in the intestines of humans and other warm-blooded animals. Most *E. coli* stains are harmless and play a pivotal role for human intestinal health, though some of them are pathogenic and can cause diseases such as diarrhea, dysentery to hosts (Donnenberg, 2002). *B. cereus* is a Gram-positive bacterium and is widespread in environments and foods. Some *B. cereus* strains are opportunistic pathogens and can cause periodontal diseases and other more serious infections (Bottone, 2010). Therefore, large numbers of *B. cereus* receiving *erm* resistance genes may increase health risks of pathogens.

The results of scanning electron microscopy (SEM) image analysis are shown in Figure 2-4. *E. coli* were rod-shape bacteria and their sizes (1-2 μm) are smaller than *B. cereus* (3-4 μm), which were rod shaped with square ends and appeared in short chains.

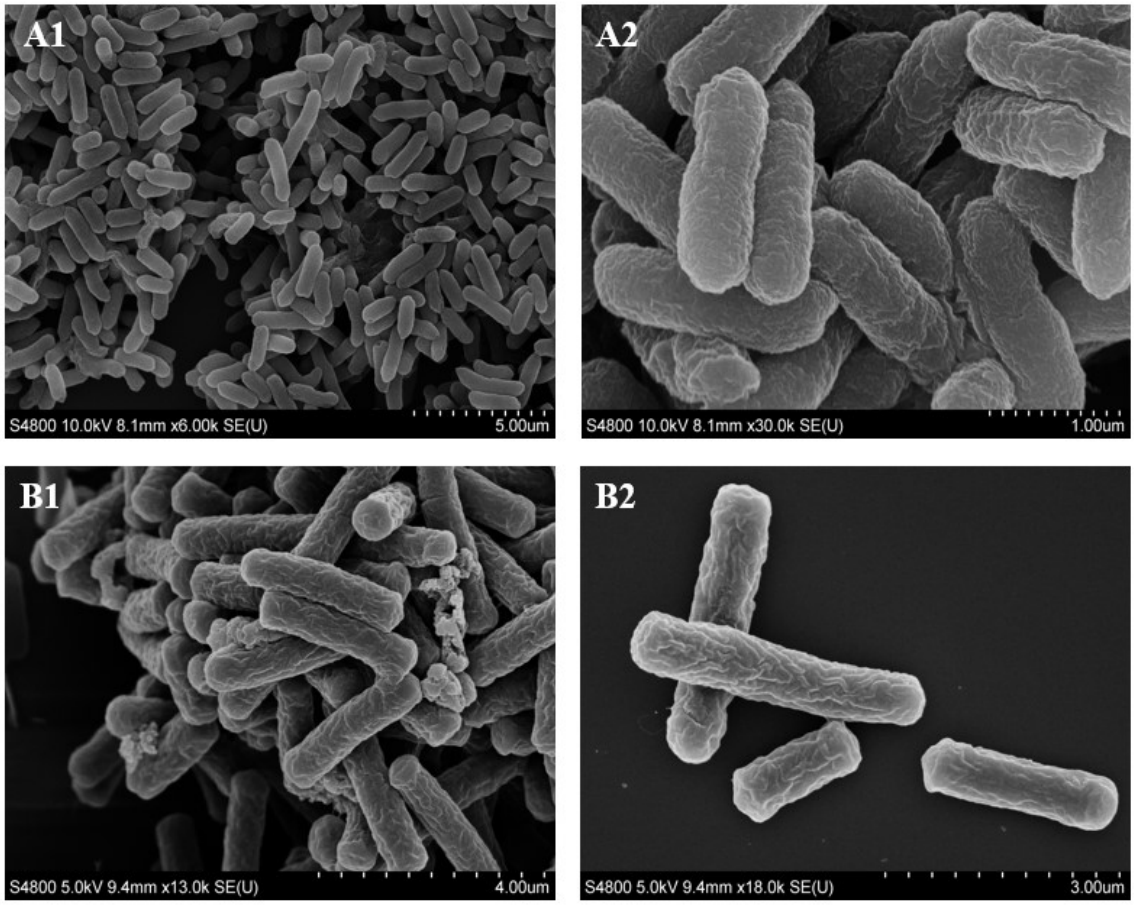

Figure 2-4 SEM images of donor and recipient strains before mating experiments. (A1) *E. coli* under 6,000 × magnification. (A2) *E. coli* under 30,000 × magnification. (B1) *B. cereus* under 3,000 × magnification. (B2) *B. cereus* under 16,000 × magnification.

2.3.2 Conjugation frequency in mating experiments.

As shown in Figure 2-5, only the cells harboring resistance *erm*80 genes could be detected by *erm*80 primers in this study. After conjugation, the sensitive recipients showed a band in the gel electrophoresis image, which indicated that *erm*80 genes had been transferred to the recipients.

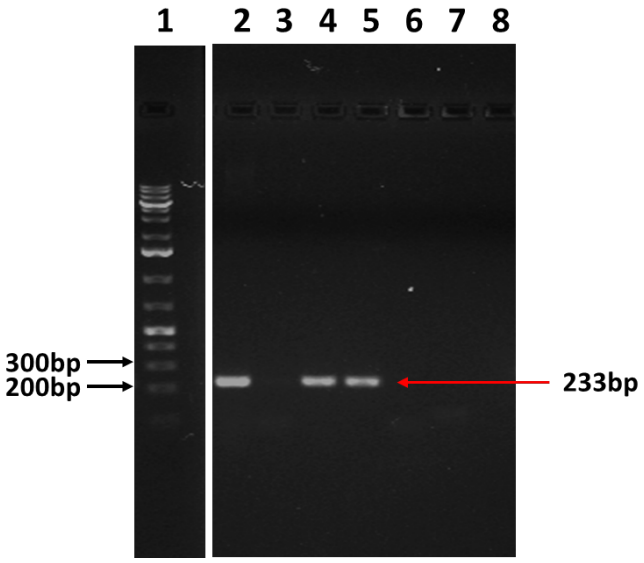

Figure 2-5 Gel electrophoresis of *erm*80 gene detected by PCR in mating experiments. Lane 1: 1kb plus DNA ladder; Lane 2: *E. coli* with pTE80 plasmid; Lane 3: *B. cereus* without pTE80 plasmid (before mating experiment); Lane 4: transconjugants under 10^{-2} × MIC erythromycin; Lane 5: transconjugants under 10^{-4} × MIC erythromycin; Lane 6: recipients under 10^{-2} × MIC erythromycin (spontaneous mutants); Lane 7: recipients under 10^{-4} × MIC erythromycin (spontaneous mutants); Lane 8: negative control (sterile water as DNA template).

Horizontal transfer of *erm*80 genes was detected in all samples after 48 hr of incubation. Results from plate mating experiments with donors, recipients, and transconjugant cells under different erythromycin concentrations are presented in Figure 2-6 to Figure 2-11. The cell numbers of cultivated recipients in control groups and recipients in mix culture under the exposure to erythromycin increased and plateaued after 24 hr of incubation, and then decreased with time. The overall growth trend of recipients with erythromycin treatments were lower than that of recipients without treatment. Similarly, the colony numbers of transconjugants (subtracted with the numbers of spontaneous mutants) significantly increased and peaked at 0 × MIC, 10^{-6} × MIC, 10^{-2} × MIC of erythromycin after 24 hr of incubation. Significantly higher numbers of transconjugants were observed in mating experiments with sub-MIC of erythromycin than those with under exposure to high levels of erythromycin (1 × MIC and 10 × MIC). No transconjugants were found under 10 × MIC within 48 hr of incubation, but those with sub-MIC erythromycin resulted in 10^2 to 10^3 CFU/mL of transconjugants. The result is consistent with several previous studies (Bahl et al., 2004; Jutkina et al., 2016; S. Kim et al., 2014), which showed that higher concentrations (above 5

mg/L) of tetracycline and streptomycin caused lower numbers of transconjugants than sub-MIC levels of antibiotics.

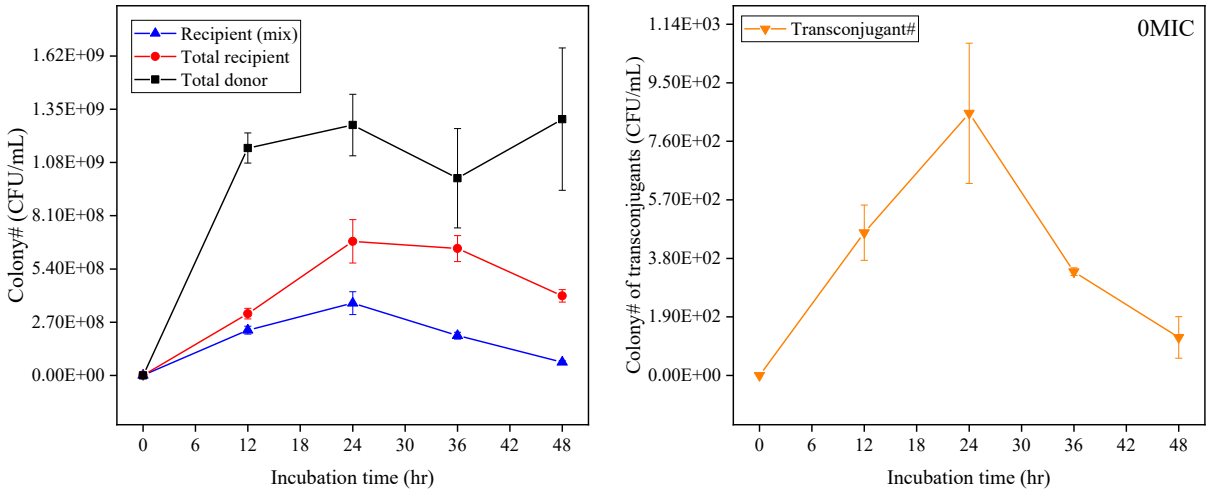

Figure 2-6 Effect of 0 × MIC erythromycin on cultivable cell numbers. Black squares: total numbers of donor grown without erythromycin; red circles: total numbers of recipient grown without erythromycin; blue triangles: numbers of recipient grown with donors; orange upside down triangles: numbers of transconjugants with *erm*80. Error bars represent standard error of three replicates.

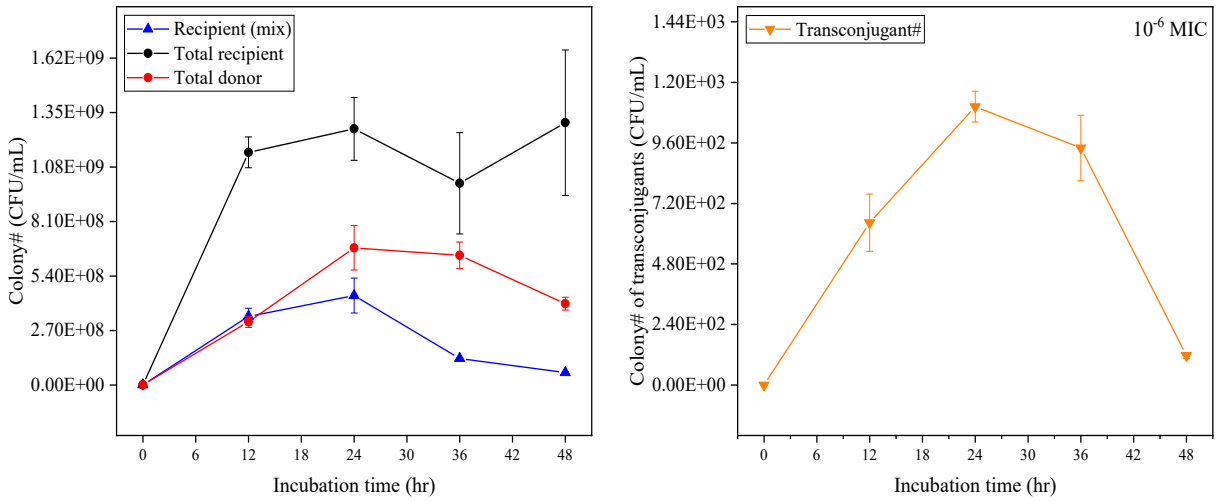

Figure 2-7 Effect of 10^{-6} × MIC erythromycin on cultivable cell numbers. Error bars represent standard error of three replicates.

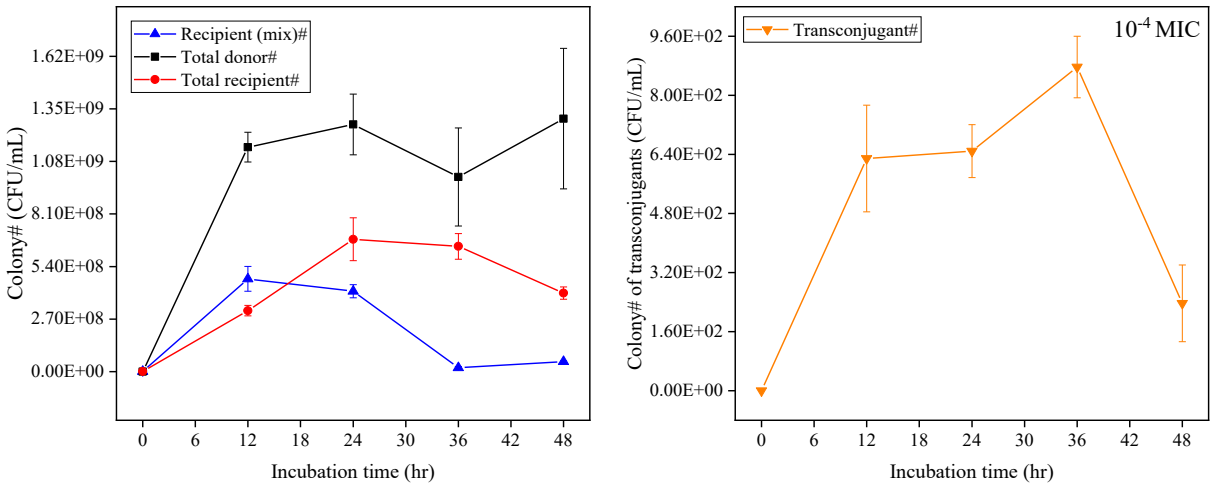

Figure 2-8 Effect of 10^{-4} × MIC erythromycin on cultivable cell numbers. Error bars represent standard error of three replicates.

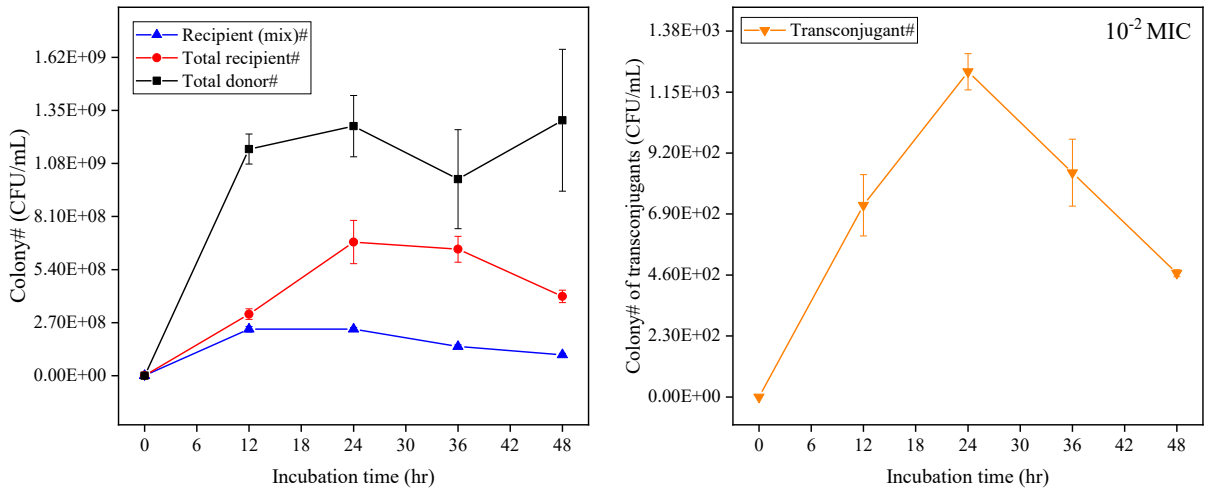

Figure 2-9 Effect of 10^{-2} × MIC erythromycin on cultivable cell numbers. Error bars represent standard error of three replicates.

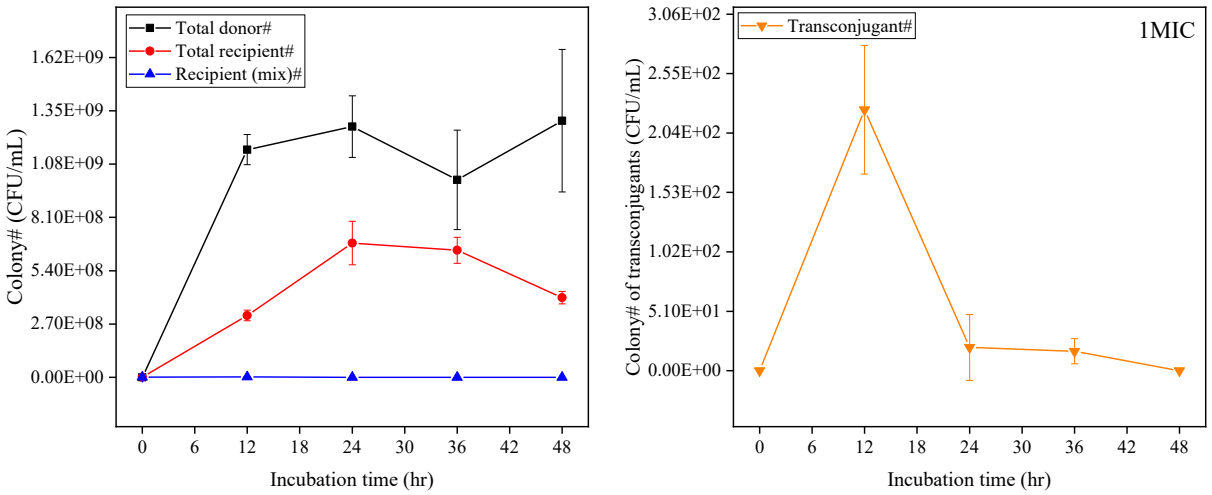

Figure 2-10 Effect of 1 × MIC erythromycin on cultivable cell numbers. Error bars represent standard error of three replicates.

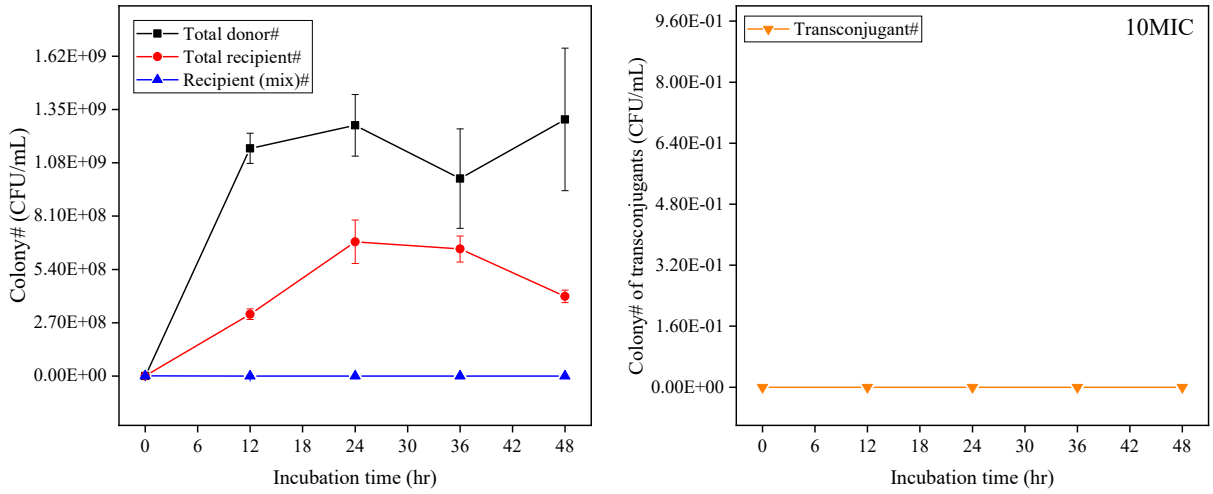

Figure 2-11 Effect of 10 × MIC erythromycin on cultivable cell numbers. Error bars represent standard error of three replicates.

In the mating experiments, transfer frequencies of *erm*80 gene were higher under the exposure to sub-MIC levels of erythromycin**.** As shown in Figure 2-12, transfer efficiency (transconjugants/total numbers of recipients) after 12 hr of incubation was the highest among other transfer efficiencies at different incubation times. In the horizontal transfer experiment, the natural transfer efficiency of *erm*80 gene in mix cultures was as low as $14.8 \pm 3.06 \times 10^{-7}$ in control groups (no erythromycin). The presence of erythromycin promoted transfer efficiency of *erm*80 resistant

gene approximately 4-fold higher than untreated control groups. The transfer frequency reached a peak value of 23.1 ± 4.20 × 10^{-7} at 10^{-2} × MIC (4 ×10^{-3} mg/L erythromycin) after 12 hr of incubation. Over the period from 0 hr to 48 hr, horizontal gene transfer of *erm*80 stayed the highest under the exposure of 10^{-2} × MIC except the efficiency at 36 hr. After 36 hr of incubation, conjugation frequency under 10^{-6} × MIC (14.6 ± 2.51 × 10^{-7}), was slightly higher than the efficiencies under 10^{-4} × MIC and 10^{-2} × MIC, which were 13.6 ± 1.90 × 10^{-7} and 13.2 ± 2.37 × 10^{-7}, respectively. Under high levels of erythromycin exposure experiments (1 × MIC and 10 × MIC), the transfer efficiency decreased with increasing concentrations of erythromycin.

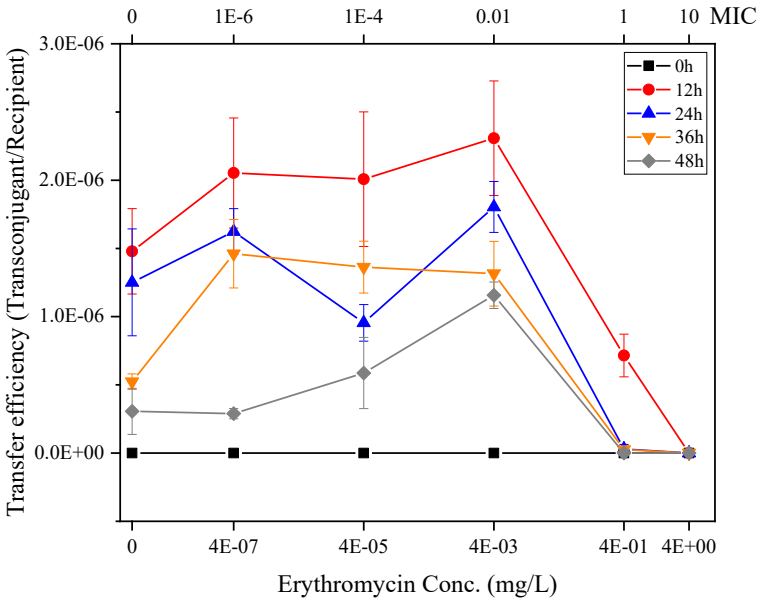

Figure 2-12 Effect of different concentrations of erythromycin on transfer frequency. Black squares: transfer frequencies after 0 hr incubation; red circles: transfer frequencies after 12 hr incubation; blue triangles: transfer frequencies after 24 hr incubation; orange upside down triangles: transfer frequencies after 36 hr incubation; grey diamonds: transfer frequencies after 48 hr incubation. Error bars represent standard error of three replicates.

The transfer efficiency of *erm* gene varied significantly among concentrations of erythromycin, suggesting that trace level of erythromycin is significant to facilitate conjugation between donor and recipient bacteria in the environment. Several previous studies have shown that low concentrations of antibiotics, which could be in the ppb level, can enhance HGT of ARGs between bacteria. One study showed that transfer frequencies were increased in 10 ppb and 100 ppb of

tetracycline and sulfamethoxazole between *E. coli* DH5α with pB10 antibiotic resistant plasmid and *Pseudomonas aeruginosa* (S. Kim et al., 2014). Another study reported that the conjugative transfer frequencies of multi-antibiotic resistance plasmid pWG613 in *Staphylococcus aureus* was enhanced by 10- to 20-fold under 500 ppb gentamicin and 1000 ppb vancomycin (Al-Masaudi et al., 1991). Even though antibiotic concentrations tested in previous studies were low, the conjugative transfer between bacteria at extremely low and environmentally prevalent antibiotic concentrations have not been investigated.

The results of the mating experiments clearly showed that transfer frequencies of *erm*80 gene at sub-MIC levels of erythromycin (10^{-2} × MIC to 10^{-6} × MIC) were always higher than those at higher concentrations (1 × MIC and 10 × MIC). Our results support that effects of antibiotics on sensitive bacteria are concentration-dependent, which are in agreement with previous studies (Goh et al., 2002; S. Kim et al., 2014; Linares et al., 2006; Yim et al., 2007). Therefore, extremely low concentrations of antibiotics in the natural environment are likely to facilitate HGT and result in more sensitive bacteria receiving ARGs. When sensitive bacteria are exposed to a high (also lethal) concentration of antibiotics, only the cells acquired the ARGs or mutants could survive, whereas the remaining sensitive cells are dead. Thus, the role of antibiotics at lethal concentrations is to inhibit or kill the donors and recipients. In contrast, growth of sensitive bacteria exposed to sub-MIC of antibiotics are inhibited not killed. Therefore, sub-MIC (non-lethal concentration) would facilitate the transfer of ARGs and result in more population of sensitive cells received ARGs (Andersson & Hughes, 2014). At non-lethal concentrations, antibiotics can even yield beneficial effects on the survival of sensitive bacteria in natural environments. The reduced numbers of recipients incubated in the mix culture at 1 × MIC and 10 × MIC of erythromycin could be employed to prove this theory. Lethal levels of erythromycin inhibited or killed the recipient cells so that the numbers of transconjugants were decreased, which means the probability of recipient cells to receive the *erm*80 genes was reduced.

Cell to cell contact by bacterial biofilm could also facilitate HGT under sub-MIC of antibiotics. Many studies have shown that sub-MICs of erythromycin, azithromycin, clarithromycin (Q. Wang et al., 2010), vancomycin, tigecycline, linezolid, novobiocin (Kaplan et al., 2011), and tetracycline (Rachid et al., 2000) could induce the formation of biofilm. Under 1/4 × MIC of erythromycin (4

μg/mL to 32 μg/mL), 20% of isolates demonstrated enhanced biofilm formation, while no biofilm formation was induced at 1/1,024 × MIC of erythromycin (Q. Wang et al., 2010). Likewise, antibiotic-induced biofilm formation was significantly increased at 3/4 × MIC of vancomycin (around 1.5 μg/mL) but decreased at lower concentrations of vancomycin (over range of 0 μg/mL to 1.5 μg/mL) (Kaplan et al., 2011). The results of biofilm formation induced by sub-MIC antibiotics could also explain the variations of transfer efficiency of *erm*80 genes in this study. 10^{-2} × MIC of erythromycin may cause the maximum biofilm induction, but lower or higher concentration than 10^{-2} × MIC may have no or reduced effects on biofilm formation between *E. coli* and *B. cereus*. Conjugation process requires cell-to-cell contact, thus biofilm between donors and recipients can help increase the conjugative transfer of ARGs since biofilm increases connection between donors and recipients (Angles, 1993; Krol et al., 2011).

2.3.3 Effect of erythromycin on bacterial viability

Bacterial growth is a main contributor to the development of antibiotic resistance. The increasing bacterial populations can enhance the possibility of bacterial survival with addition of antibiotics, thus the frequency for the cells to receive ARGs would be increased.

To confirm the concentration-dependent theory above, the donor strain (*E. coli*) and the recipient strain (*B. cereus*) were separately incubated with various concentrations of erythromycin to measure the viability rate. The overall bacterial growth of donor and recipient was inhibited after exposure to erythromycin. The viability rates of donor and recipient under different concentrations of erythromycin are shown in Figure 2-13. With an increase of erythromycin concentration, the viability rate of donor and recipient decreased. When the concentrations of erythromycin were 0.4 mg/L and 4 mg/L, which were the lethal concentration for recipients, the recipient bacteria were significantly inhibited. These data indicated that altered concentrations of erythromycin did not significantly change the numbers of donors, except in the absence of erythromycin. Therefore, the decreased number of transconjugants at 1 × MIC and 10 × MIC of erythromycin can be attributed to the reduced viability rate of recipient.

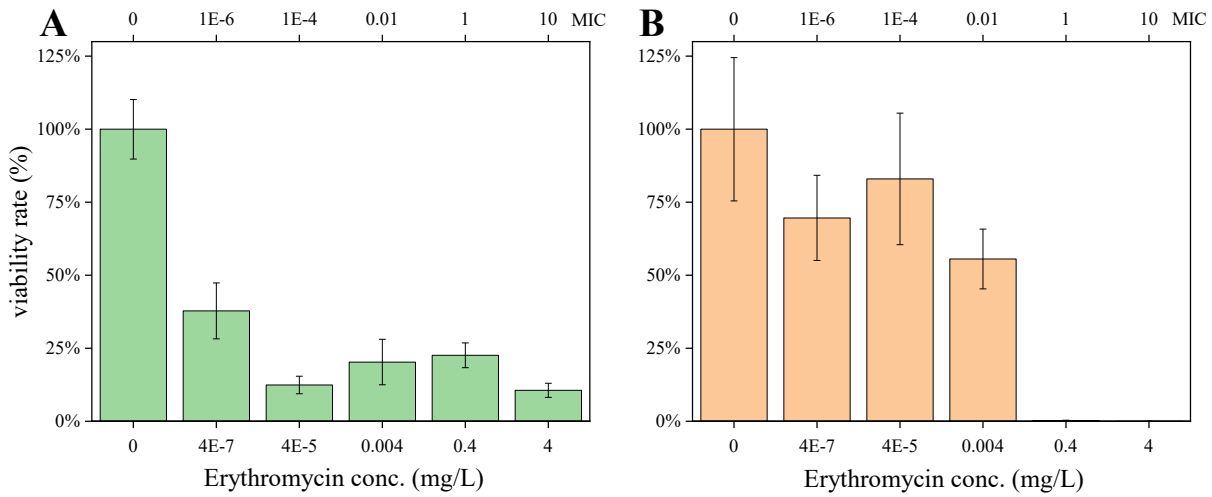

Figure 2-13 Effect of erythromycin on bacterial viability of *E. coli* and *B. cereus*. (A) *E. coli* exposed to 0 mg/L, 4×10^{-7} mg/L, 4×10^{-5} mg/L, 4×10^{-3} mg/L, 0.4 mg/L and 4 mg/L of erythromycin. (B) *B. cereus* exposed to 0 mg/L, 4×10^{-7} mg/L, 4×10^{-5} mg/L, 4×10^{-3} mg/L, 0.4 mg/L and 4 mg/L of erythromycin. The values indicate the mean of triplicate experiments, and error lines represent the standard error of triplicate experiments.

2.3.4 Abundance of *erm*80, *intI*1, and 16s rRNA genes

The gene copy numbers of 16S rRNA (mean $107.0 \pm 2.2 \times 10^7$) did not differ between different antibiotic concentration levels over a period of 48 hr (except 0 hr) as shown in Figure 2-14. The copy numbers of 16S rRNA gene were lowest at the beginning (0 hr) and reached peaks at 12 hr except for 10 × MIC erythromycin group. The trend of 16S rRNA concentration was towards declining at the later hours, indicating a reduction of cells. The reasons for the declining numbers of bacteria could be low nutritional condition, lack of oxygen, antibiotic, or other environmental factors. To maintain the antibiotic resistance function may be too costly for bacteria, leading to their death. It may explain the stable *erm*80 concentration during the whole incubation time, while the copy numbers of 16S rRNA declined. Under 10 × MIC selective pressure, there was a delay on the growth of 16S rRNA genes within the first 12 hr (Figure 2-14 F). Then the gene copy numbers of 16S rRNA increased from $15.2 \pm 1.4 \times 10^7$ to $118.0 \pm 2.4 \times 10^7$ gene numbers/mL over the range from 12 hr to 48 hr, suggesting that higher concentration of erythromycin (> 1 × MIC) could cause delay in the bacterial growth.

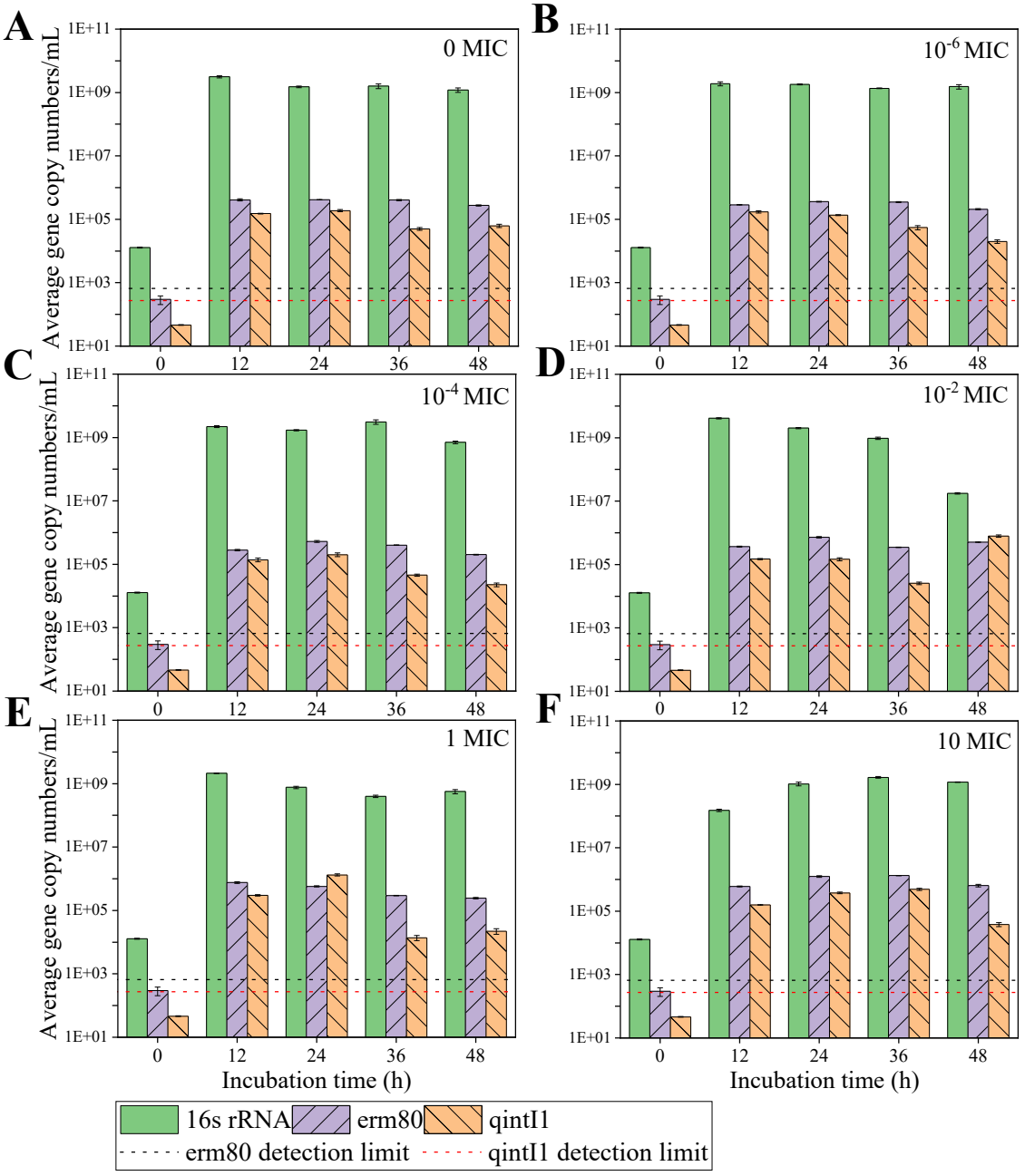

Figure 2-14 Effects of different concentrations of erythromycin on gene abundances. The concentrations of erythromycin exposure in panel A to F are 0, 4×10^{-7}, 4×10^{-5}, 4×10^{-3}, 0.4, 4 mg/L, respectively. Error bars represent the standard error of three replicates.

The abundances of *erm*80 genes did not significantly vary either with different concentrations of erythromycin (mean value was $698.0 \pm 4.0 \times 10^3$ gene copies/mL) or over time, indicating that the concentrations of erythromycin and incubation time did not affect the gene copy numbers of *erm*80. This case could be explained by the viability of donor cells, as the concentrations of erythromycin

did not significantly affect the growth of donor cells harboring the *erm*80 genes. In the total bacterial community, the donors and tranconjugants with *erm*80 genes became dominant under lethal concentrations of erythromycin, while the sensitive recipients were inhibited by the erythromycin. There was a lot of similarities between the trend of *erm*80 abundance and that of *intI*1 abundance, which will be discussed later in next session.

SOS induction related to conjugative transfer was detected in the total bacterial population by quantifying the gene copy numbers of *intI*1 genes and *erm*80 genes (Figure 2-14). When the recipient cells were mixed with the conjugation proficient donor cells at 0 hr, very few *intI*1 genes (46.3 gene copies/mL) were detected in the mix culture. Much higher gene copies of *intI*1 genes (4.0×10^5 gene copies/mL) were observed after 12 hr to 48 hr of mating with donors, suggesting that SOS response was induced with erythromycin treatment. Multiple antibiotic resistances in *E. coli* have been shown to be associated with mobile genetic elements like integrons (Bass et al., 1999; Oberle et al., 2012). Integrons are also considered as a main part for bacteria acquiring ARGs through HGT from other bacteria. To verify that the occurrence of *intI*1 genes was related to the horizontal transfer of *erm*80 genes, we investigated the correlations between the transfer of *erm* genes and the occurrence of *intI*1 genes.

As shown in Figure 2-14, 16S rRNA, *erm*80, and *intI*1 genes reached peaks between 12 hr and 24 hr of mating experiments. Then the numbers of those genes reduced at sub-MIC erythromycin from 36 hr to 48 hr, except the data under 10 × MIC. This can be explained by the growth curves of donor and recipient cells. At the initial stage of mating, the plasmid with *erm*80 genes entered the recipient cells, triggered the SOS response, and the recipient that acquired *erm*80 genes multiplied. Therefore, the population of erythromycin resistant host cells increased during 24 hr. Accordingly, the reductions of *erm*80 and *intI*1 genes were related to the decreasing numbers of host cells. Once the increasing amount of the cells have acquired the ARG, the SOS response would diminish (Baharoglu et al., 2010). Consequently, the number of *intI*1 genes decreased between 36 hr and 48 hr of mating.

Although fewer or no transconjugants were detected by conventional culture-based method at 1 × MIC and 10 × MIC, no significant reduction of the gene copy numbers of 16S rRNA, *erm*80 and

*intI*1 genes was quantified by qPCR. This result can be explained by the presence of VBNC cells at lethal level of antibiotics. Although the gene abundance was not significantly affected by the cultivability of bacteria cells, it is impossible for qPCR to differentiate the donor, recipient and transconjugants in mating experiments. Hence, it is necessary to employ FISH method to detect the *erm* gene expression, which is not affected by the cultivability of bacterial cells and also calculate the rates of *erm* gene resistance.

2.3.5 Correlation between the abundance of *erm*80 and *intI*1 genes

The changes of ARG abundance were tracked with *erm*80 genes and *intI*1 (Figure 2-15).

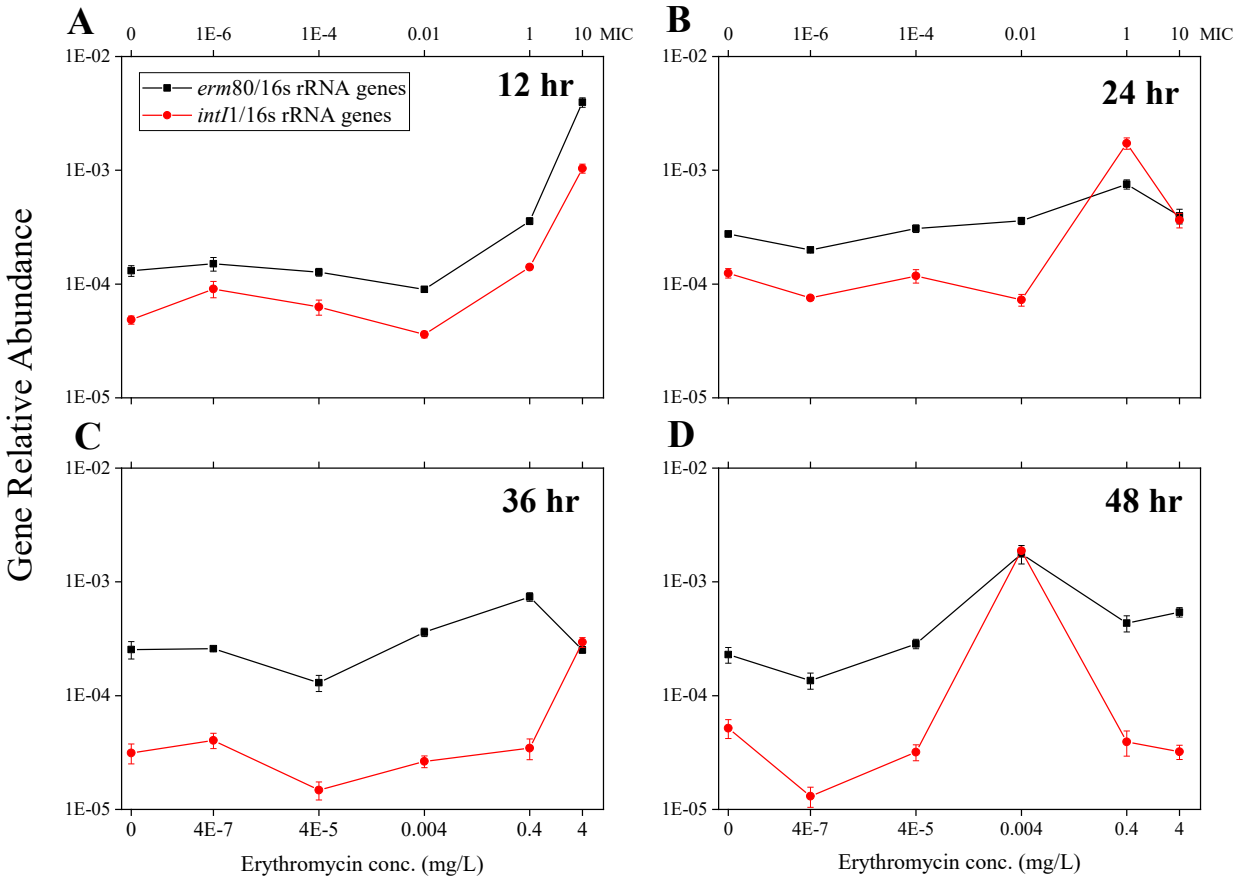

Figure 2-15 Correlation between relative gene abundances of *erm*80 and *intI*1. Black squares: gene relative abundance of *erm80* genes; red circles: gene relative abundance of *intI*1 genes. Error bars represent standard error of three replicates.

The increasing abundance of *erm*80 was consistent with the increasing abundance of the *intI*1 genes, indicating that the proliferation of *intI*1 genes, which was promoted by sub-MIC antibiotics, resulted in horizontal transfer of ARGs. Many ARGs in bacteria, especially Gram-negative bacteria, are associated with mobile genetic elements, which have the ability to carry different DNA molecules and thus transfer them among different cells by conjugation (Partridge et al., 2009). Plasmids along with integrons have been found to be transferred to diverse bacteria that belonging to eleven different phyla (Klümper et al., 2015). Antibiotics at non-lethal concentrations have been demonstrated to induce mobilization of bacterial genetic elements and enhanced the transfer frequency. For example, integrase expression was promoted with addition of trimethoprim, quinolones, and β-lactams (Guerin et al., 2009). Another example is that conjugation transfer of SXT induced SOS response in *V. cholerae* as recipient and increased 12-fold of *intI*1 genes compared to control without SXT plasmid (Baharoglu et al., 2010). In conclusion, the SOS response by extremely low concentrations of erythromycin triggers the expression of *intI*1 genes, resulting in the conjugative transfer of *erm*80 genes in the process of HGT.

The selection of antibiotic resistance for bacteria or pathogen is also determined by their fitness costs. The fitness cost of resistance would allow sensitive bacteria to out-compete resistant bacteria when the selective pressure is decreased (Gullberg et al., 2011). It is costly for bacteria to host the resistance mechanisms, and consequently making these bacteria with selective disadvantages and lose their viability in the absence of antibiotics Therefore, antibiotic selected bacteria that experience low or no fitness cost are more likely to grow without antibiotics pressures (Andersson, 2006). Studies have reported that the ARGs in integrons can remain silenced at no biological cost, but other adaptive functions still activated at the same time (Melnyk et al., 2015). This characteristic of this mechanism is also responsible for the dissemination of bacteria harboring hidden resistance determinants (Foucault et al., 2010). Several studies have illustrated the fitness cost associated with mobile genetic elements. No fitness cost was imposed on *E. coli* 345-2 Rfic to acquire an ampicillin resistant element (Tn1) and a trimethoprim resistant element (Tn7) (Enne et al., 2005). A similar study showed that the acquisition of a conjugative transposon Tn1649 had no fitness cost for *Enterococcus faecium* and *Enterococcus faecalis* (Foucault et al., 2010). When the concentration of antibiotic is higher than the MIC, the main driving force of the selection would be antibiotic resistance and the fitness cost would be less important due to the death of sensitive

bacteria. When the concentration of antibiotic is lower than the MIC, the situation is different since the growth of sensitive bacteria is not completed inhibited, they only grow slower. Therefore, resistant selection conferring low fitness cost in the sensitive bacteria would like to be grown at sub-MIC levels of antibiotics. These results suggest that sub-MIC antibiotics as a selective pressure would be a crucial contributor to select for antibiotic resistance and ARGs in natural environments where the concentration of antibiotics are under sub-MIC levels.

2.3.6 MLS_B resistance in conjugation and correlation with sub-MIC of erythromycin

FISH was used for an accurate identification of the methylation of 23S rRNA resulted from expression of *erm* genes in recipient strain. Before acquisition of the *erm*80 genes for *B. cereus*, all cells hybridized with MLS_B sensitive probes showed red-color in FISH images. After mating experiments, *erm*80 genes were transferred to some *B. cereus* that could not be hybridized with MLS_B probes (Figure 2-16). All cells were stained with DAPI, which showed blue color in FIGH image, and erythromycin sensitive cells hybridized with red MLS_B probes, while resistant cells hybridized with sensitive probes showing no color in FISH images.

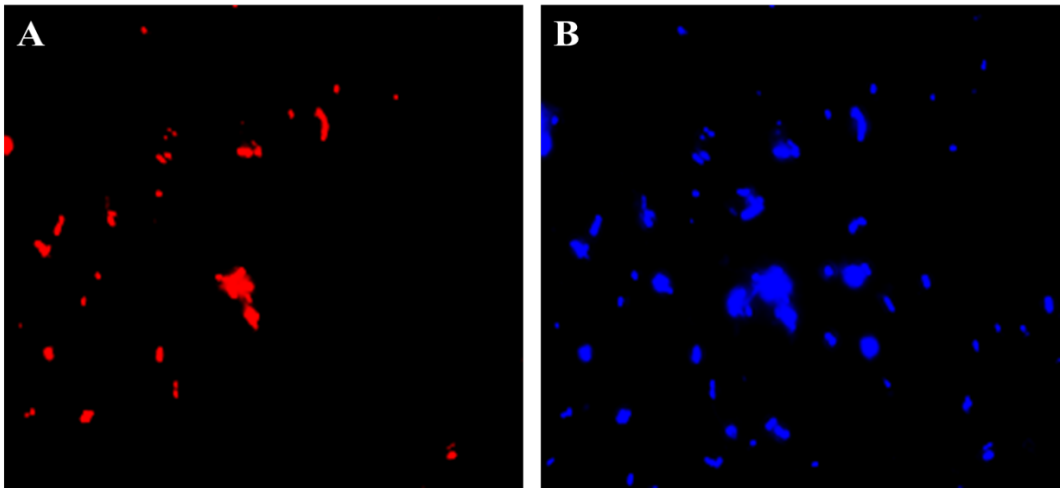

Figure 2-16 Sample FISH images. (A) cells were hybridized by MLS_B sensitive probe. (B) cells were stained with DAPI.

Since erythromycin can strongly induce *erm* gene expression, high levels of MLS_B resistance was observed (Figure 2-17). This result is in agreement with previous study (Zhong et al., 1999) that levels of 23S rRNA methylation can be triggered by erythromycin and azithromycin. The rates of

MLS_B-resistant bacteria with the expressed *erm* genes were highest at 91.2% under 10^{-2} × MIC treatment, consistent with our previous transfer efficiency measured by culture-based method. Correlation between the rates of MLS_B resistance measured by FISH and erythromycin concentrations was strong, especially at 1 × MIC and 10 × MIC erythromycin. The rates of MLS_B resistance at 10 × MIC erythromycin increased from 16.5% to 35.6% after 48 hr of incubation, and no transfer frequency was found at 10 × MIC over 48 hr period of incubation time by culture-based method. These results indicated that higher level of antibiotic, such as 10-fold higher than the MIC, may play a significant role in the horizontal transfer of ARGs. Additionally, methylation as a result of *erm* gene expression, instead of colony formation, was a better indicator of selective pressure of high levels erythromycin.

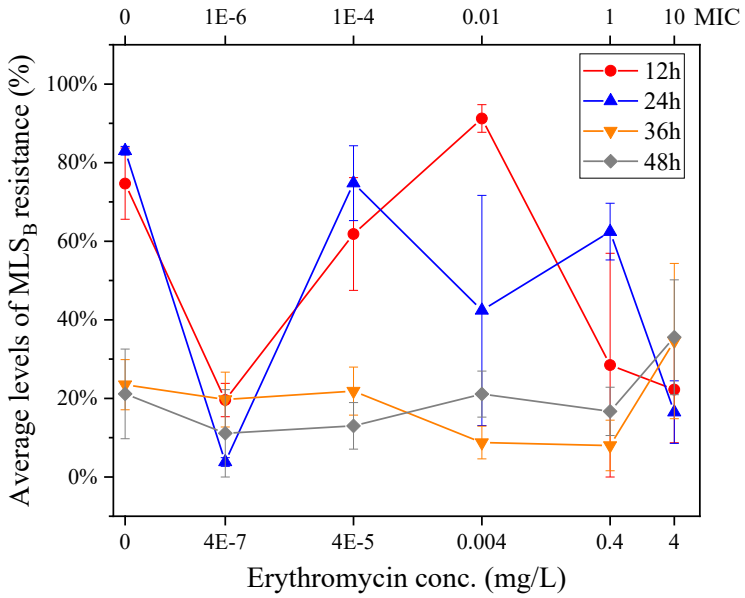

Figure 2-17 Effect of erythromycin on MLS_B resistance in mating experiments. Red circles: percentage of MLS_B resistance after 12 hr incubation; blue triangles: percentage of MLS_B resistance after 24 hr incubation; orange upside down triangles: percentage of MLS_B resistance after 36 hr incubation; grey diamonds: percentage of MLS_B resistance after 48 hr incubation. Error bars represent the standard error of three replicates.

We also identified that nonlethal levels of erythromycin had a strong influence on the induction of 23S rRNA methylation, which was the product of *erm* genes. The overall level of MLS_B resistance was from 3.8% to 91.2% in mating experiments, with similar trends of *erm*80 gene transfer frequency. Another study illustrated that the environmentally relevant concentrations of antibiotics

were significantly related to the prevalence of ARGs quantified by culture-based method in aquatic systems of northern Vietnam (Hoa et al., 2011). Similar results were previously observed (Heß et al., 2014), which reported that the induction of resistance to MLS_B by erythromycin at 1 ng/L concentration via qPCR. Another study indicated that no noticeable influence of low level of antibiotics (a much smaller range from 100 to 2000 ng/L compared with ours) on the abundance of ARGs detected by qPCR in surface-flow constructed wetlands (Berglund et al., 2014). However, the temperatures they applied (between 8.6 ºC and 17.1 ºC) were not the favorable growth condition for many bacteria, which might be the limiting factor than the antibiotic concentration. Overall, the influence of sub-MIC erythromycin on the horizontal transfer of *erm*80 genes was confirmed. The similarities of culture-based and FISH results further validated the accuracy of FISH methods.

The numbers of transconjugants measured by FISH (Figure 2-18) were significantly higher than that tested by culture-based method (Figure 2-19 to Figure 2-11).

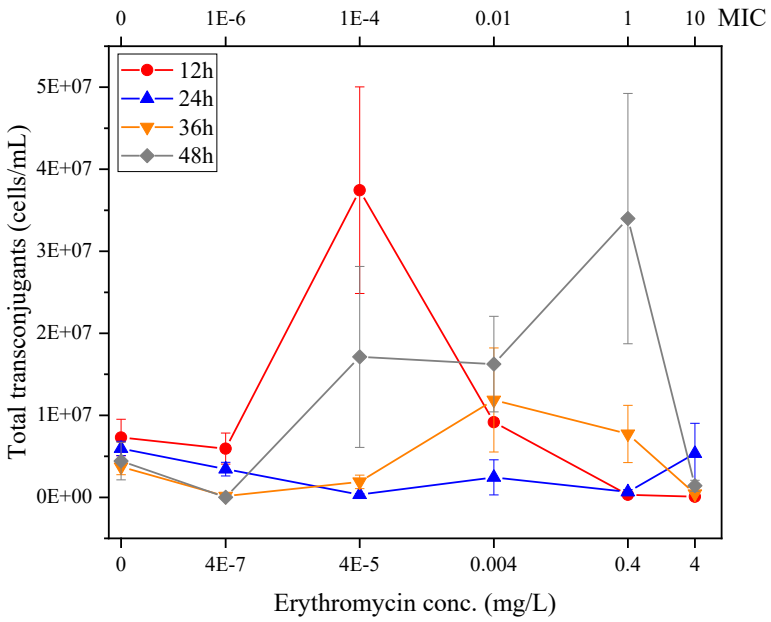

Figure 2-18 Effect of erythromycin on total transconjugants quantified by FISH. Red circles: total cell numbers of transconjugants per mL after 12 hr incubation; blue triangles: total cell numbers of transconjugants per mL after 24 hr incubation; orange upside down triangles: total cell numbers of transconjugants per mL after 36 hr incubation; grey diamonds: total cell numbers of transconjugants per mL after 48 hr incubation. Error bars represent the standard error of three replicates.

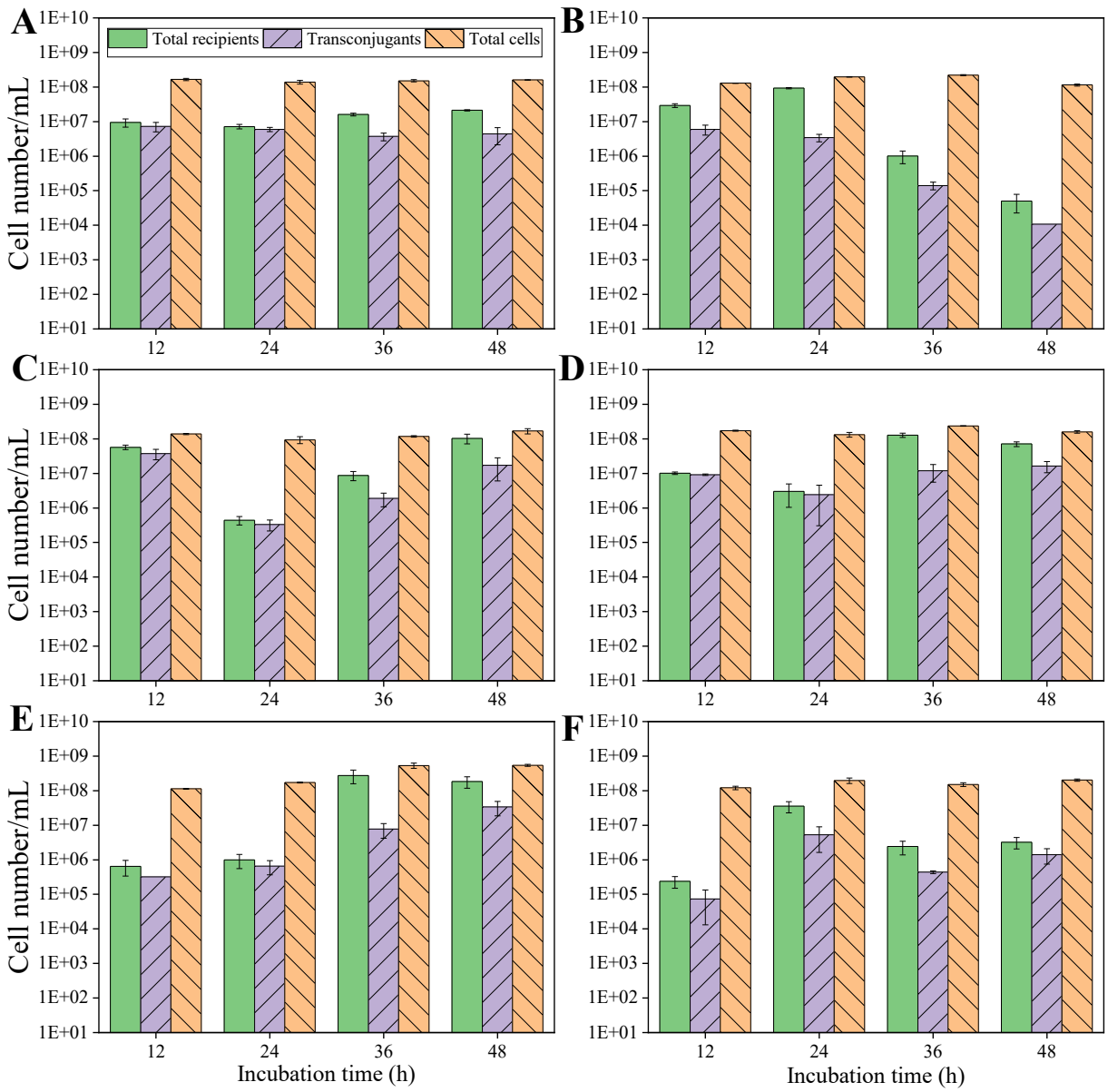

Figure 2-19 Cell numbers of donor, recipient, and transconjugants quantified by FISH. The concentrations of erythromycin were (A) 0 mg/L, (B) 4×10^{-7} mg/L, (C) 4×10^{-5} mg/L, (D) 4×10^{-3} mg/L, (E) 0.4 mg/L, (F) 4 mg/L. Error bars represent the standard error of three replicates.

With the presence of antibiotics, especially at 10 × MIC erythromycin concentration, 10^5 to 10^6 cells/mL of transconjugants were observed from FISH, whereas no colony was formed at the same concentration of erythromycin by culture-based method. The difference was due to the limitation of the culture-based approach, failing to identify any cells that entered the VBNC under the

presence of erythromycin to survive. FISH results further confirmed that the fitness cost driven by antibiotics was concentration-dependent, consistent with the fact that the VBNC state was one of the adaptive ways to survive, therefore depending on the antibiotic concentrations. Without antibiotics, the VBNC state still came into effect in the culture-based approach due to the competing growth between donors and recipients in a common LB media, confirmed by the smaller amount of recipient colonies observed when both donors and recipients were present compared to the case of recipients alone.

The lack of nutrients is also common in most natural environments. A previous study showed that conjugation process can appear in bacteria when the environments containing little to no organic carbon which obviously cannot select for antibiotic resistance plasmids (Chandrasekaran et al., 1998). The study mixed *Pseudomonas fluorescens* harboring a multi-resistance plasmid with four marine isolates in LB broth. The conjugal transfer frequency in broth and plate mating experiments was as high as 10^{-3}. In another mating test performed in natural seawater without any nutrients, the frequency of plasmid transfer was from 10^{-4} to 10^{-7} from donor to four marine bacteria. After the donor cells have become VBNC state, they also obtained transfer frequencies of 10^{-5} to 10^{-7} indicating that nutrient starvation and the VBNC state may not restrict conjugal transfer between donors and recipients.

It is apparent that sub-MIC antibiotics not only affect genetic changes of bacteria but also impact bacterial metabolic activities. Some studies showed that *E. coli, Staphylococcus aureus*, *Legionella pneumophila* could enter the VBNC state when exposed to antibiotics, low-level chlorine and chlorination (Dusserre et al., 2008; Lin et al., 2017; Pasquaroli et al., 2013). Bacteria in VBNC state are not able to grow on conventional microbiological media which they normally grow into colonies, but still keep alive and maintain their metabolic potential (James D. Oliver, 2005). Their biological functions are not totally lost or impaired as well. Gene expression does not terminate and plasmids are still retained. A study demonstrated that *E. coli* cells in the VBNC state was still able to express antibiotic resistance plasmid (Bale et al., 1988). Even the bacteria treated with lethal level of erythromycin which lose cultivability can enter into the VBNC state and are still able to express *erm* genes when environments become appropriate. Therefore, it is advisable to

adopt FISH method instead of culture-based approach especially when the VBNC state can be a significant effect.

2.4 Conclusion

Numerous studies have reported the positive relationships between antibiotics and emergence of ARGs in nature environment. However, few studies exist regarding effects of sub-MIC level of antibiotics on the horizontal transfer of ARGs between bacteria and pathogen, especially at concentrations of the natural environment. The study showed that even at a low erythromycin concentration of 4×10^{-7} mg/L, conjugative transfer frequency of *erm*80 genes was still heavily enhanced by the presence of erythromycin. The highest enhancement occurred at 4×10^{-3} mg/L of erythromycin.

This study compared multiple approaches to detect the occurrence and development of ARGs. The traditional culture-based approach significantly underestimates the HGT process as it neglects VBNC bacteria, even commonly found in the treatment at lethal level erythromycin. The qPCR method cannot identify the HGT process. The newly designed FISH probe is capable of detecting *erm* gene expression including VBNC, accurately assessing the potential hazard of the HGT process.

The increased horizontal transfer frequency of *erm*80 genes was observed even with extremely low concentrations of erythromycin, which is contributed by the induction of the SOS response. The resulting integron stimulates conjugative transfer of *erm*80 genes. Additionally, fitness cost and biofilm formation by the sub-MIC of erythromycin also increase ARG transfer frequency.

CHAPTER 3. DEVELOPMENT OF ANTIBIOTIC RESISTANCE AND HORIZONTAL GENE TRANSFER UNDER EXPOSURE TO MULTI-WALLED CARBON NANOTUBES

3.1 Introduction

Multi-walled carbon nanotubes (MWCNTs) have been widely studied in environmental fields (Arias et al., 2006; Brady-Estévez et al., 2010), agricultural and biomedical areas (Martin et al., 2003) due to their unique physical and chemical characteristics. Particularly in environmental applications, MWCNTs were mostly studied as adsorbents to remove various organic and inorganic pollutants (G. C. Chen et al., 2009; X. Wang et al., 2009; S. Zhang et al., 2009). Many studies have also shown that MWCNTs possessed great potential for absorbing antibiotics, such as sulfonamide (Ji et al., 2009), amoxicillin (Mohammadi et al., 2014), lincomycin, and sulfamethoxazole (H. Kim et al., 2014).

With the extensive studies of MWCNTs, the toxicity of MWCNTs has been gradually recognized. Toxicity studies on MWCNT showed that they presented cytotoxicity to mammalian cells (Wick et al., 2007), and exerted strong antibacterial activities on a broad spectrum of bacteria: *E. coli*, *E. faecalis*, *B. adolescentis*, *L. acidophilus*, *S. aureus* (H. Chen et al., 2013; Kang et al., 2008). The toxicity of MWCNTs were influenced by many factors, such as the diameter and the length of MWCNT (Kang et al., 2008), impurity, the degree of dispersion (Wick et al., 2007), and also concentrations. Based on the antimicrobial activity of MWCNTs, they were also applied for inhibiting biofilm and biofouling formation (Dong et al., 2014; Malek et al., 2016).

In addition, MWCNTs were also reported to have the ability to interact with DNA due to physical absorption (Zhao et al., 2007). We already concluded that selective pressure like antibiotics could facilitate the horizontal transfer of ARGs. Therefore, the addition with MWCNTs may help to control the antibiotics and also deal with the propagation and development of ARGs. To the best of our knowledge, this is the first work that investigate the effects of sub-MIC antibiotics along with the presence of MWCNTs on the horizontal gene transfer of ARGs.

In this study, we evaluated the selective effect of MWCNT as well as one clinically relevant antibiotic on the conjugative transfer of ARGs from *E. coli* to *B. cereus* pathogen. Specifically, we (1) investigated the bacterial viabilities of *E. coli* and *B. cereus* under various concentrations of MWCNT and erythromycin, (2) evaluated the influence of MWCNT with low (50 ppm) and high (500 ppm) concentrations on the conjugative transfer of *erm* genes and mobile genetic elements, (3) evaluated the combination effects of MWCNT and antibiotics under nonlethal and lethal levels on the horizontal transfer of ARGs.

3.2 Materials and Methods

3.2.1 Preparation of CNT suspension in growth medium

C-grade multi-walled carbon nanotubes (Nanotechlabs, Yadkinville, NC) with 100 μm average length were dispersed in 0.1% Tween 80 solution and the suspension was sonicated for 15 min with the amplitude at 20% by a sonic dismembrator (Fisher Scientific, Hampton, NH) to make 5 g/L MWCNT stock solution for further dispersion in culture media.

3.2.2 Bacterial cultivation and viability test

Gram-negative bacteria *E. coli* with erythromycin resistant *erm*80 gene and Gram-positive bacteria *Bacillus cereus* were selected as donor and recipient strains in this study. Both strains were inoculated in LB broth and incubated overnight at 37 °C for further use. Different concentrations of erythromycin and MWCNT were added to donor and recipient bacterial suspension to make the final concentrations as shown in Table 3-1.

3.2.3 Conjugation experiments treated with erythromycin and MWCNT

Mating experiments were used to calculate the conjugative transfer efficiencies under exposure of erythromycin and MWCNT. Methods and antibiotic concentrations and MWCNT were described previously.

3.2.4 Gene quantification by real-time PCR

All qPCR reactions were performed on a CFX96 Real-time PCR detection system (Bio-rad, Hercules, CA), and the quantification methods, thermal cycling conditions, primers, and chemical concentrations for each reaction were described previously**.**

Table 3-1 Concentrations of erythromycin and MWCNT used in this study.

Erythromycin Conc. (mg/L)	Corresponding MIC	MWCNT Conc. (ppm)
0	0	0 50 500
4×10^{-7}	10^{-6}	0 50 500
4×10^{-5}	10^{-4}	0 50 500
4×10^{-3}	10^{-2}	0 50 500
0.4	10^{0}	0 50 500
4	10^{1}	0 50 500

3.2.5 Morphology observation of bacteria by SEM

The morphological changes of donor and recipient bacteria after incubation with MWCNT solution were observed by SEM. One mL of bacterial suspensions were condensed by centrifugation at 13,200 rpm for 10 min. The supernatant was discarded, and the pellet was washed with 0.1M phosphate buffer twice. To fix the cells, the pellet was resuspended in 2.5% glutaraldehyde and incubated for 1 hr at 4 °C. The cells were dehydrated by sequential treatment with 50, 70, 80, 90 and 100% ethanol for 10 min each time. 20 µL of dehydrated bacterial suspension was placed on a silicon chip and air dried at room temperature. The silicon chips were then mounted onto a SEM specimen stub with a carbon conductive adhesive tape, then sputter-coated with platinum (60 s, 40 mA) and viewed using a Hitachi S-4800 field emission SEM. Average length and diameter of the cells were measured by ImageJ software (https://imagej.nih.gov/ij/) based on the SEM images.

3.2.6 Cell membrane integrity Assay

3.2.6.1 Measurement of optical density at 260nm

When the membrane of bacteria is compromised, the amount of DNA and RNA released from cytoplasm of the cells can be determined by measuring the optical density at 260 nm. The bacterial suspensions treated with different concentration of erythromycin and MWCNT were incubated for 4 hr and samples of 1 mL were immediately filtered with 0.2 μm syringe PTFE filters to remove the cells. The optical density at 260 nm of samples were recorded by NanoDrop 2000c UV-Vis Spectrophotometer (Fisher Scientific, Hampton, NH) according to the manufacturer's instructions.

3.2.6.2 Analysis of bacterial viability and cell membrane permeability

While plate counting yields the quantity of culturable cells, fluorescence microscopy with Propidium iodie (PI) and SYBR Green I was employed to detect bacterial activity and cell membrane permeability. SYBR Green I is a green-fluorescent dye which can stain both live and dead cells, while PI is a red-fluorescent permeant dye which stains only injured cells with a compromised membrane. All cells with intact membranes and damaged membranes could be observed and quantified under fluorescence microscopy as a function of fluorescence intensity. Meanwhile, the plate counting method was applied to determine the viable count of the *E. coli*.

One mL of bacterial suspension from MWCNT-treated and control groups were centrifuged to pellet the cells and remove the supernatant. The pellet was resuspended in 1 × PBS buffer. Bacterial samples were stained with 1 μl of SYBR Green I (1:10,000 final dilution) and 10 μl of PI (10 μg/mL final concentration). After 30-minutes incubation in the dark, 10 μl stained samples were dropped between a glass hemocytometer (Hausser Scientific, Horsham, PA) and the coverslip to detect the cell membrane permeability and bacterial viability. The slide was viewed under the fluorescence microscope (Nikon, Melville, NY) and the cells were located under the 40 × magnification through the FITC filter and TRITC filter with an exposure time of 900 milliseconds and 800 milliseconds, respectively. 15 representative images were taken for each sample. The numbers of cells stained with fluorescence dyes were determined by direct cell counting, and cells stained with SYBR Green I were shown in green color and cells with PI were shown in red color. The percentage of cells with compromised membrane was calculated as the ratio of the number of cells stained with PI divided by the number of cells stained with both PI and SYBR Green I.

3.3 Results and Discussion

3.3.1 Physicochemical characterization of MWCNT

The average length of the MWCNT utilized in this study was 100 μm according to the manufacturer. SEM image of dispersed MWCNT is shown in in Figure 3-1. Although MWCNT with 0.1% Tween 80 solution was well dispersed via sonication procedure, large MWCNT aggregates were observed after incubating with cells. This is a common phenomenon for CNTs, and some studies reported that CNTs were aggregated to micron-size structures after incubation with cells (Kang et al., 2007; Wick et al., 2007), which may affect CNTs' toxicity. The aggregation of MWCNT-cell may be explained by van der Waals force between cell surface and MWCNT, which caused MWCNT to interact with cells and aggregate into bundles (Arias & Yang, 2006; Ji et al., 2009).

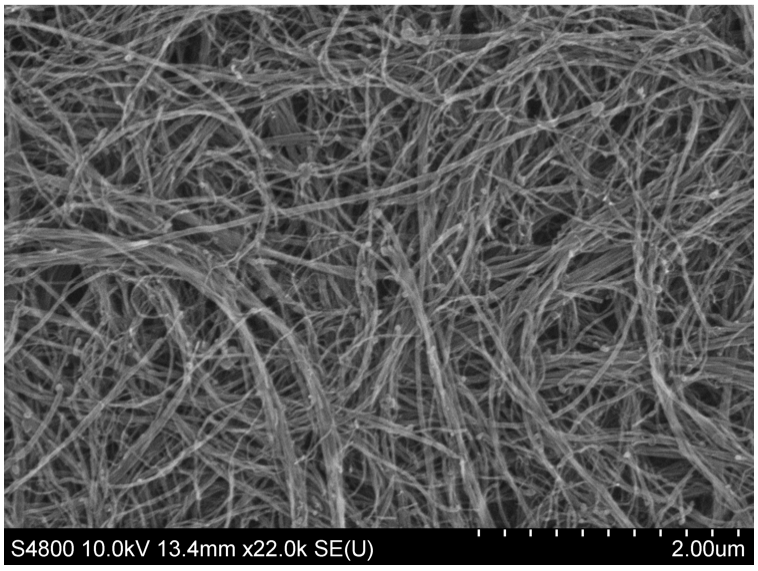

Figure 3-1 SEM image of MWCNT dispersed suspension. The MWCNT with 0.1% Tween 80 solution was well dispersed by sonication method.

3.3.2 Influence of erythromycin and MWCNT on bacterial growth

When recipient cells were challenged with 1 × MIC and 10 × MIC of erythromycin, the viability experienced a sudden decrease. Treatment with 50 ppm MWCNT produced significant positive effects on *E. coli* growth at sub-MIC, but minor effects at MIC or higher. MWCNT with 500 ppm

produced smaller effects on the growth of donor compared the effects with 50 ppm, except a relatively higher viability rate at 10 × MIC. Overall, *E. coli* bacterial growth were promoted after exposure to MWCNTs. Figure 3-2 indicated that MWCNT induced an antibacterial activity against the growth of *B. cereus* except in the absence of erythromycin. Erythromycin is the most significant impacting factor on the growth of *B. cereus*, with an obvious killing effect at 1 × MIC and 10 × MIC. In conclusion, the numbers of viable bacteria treated with different erythromycin and MWCNT may affect the transfer frequency of *erm*80 genes.

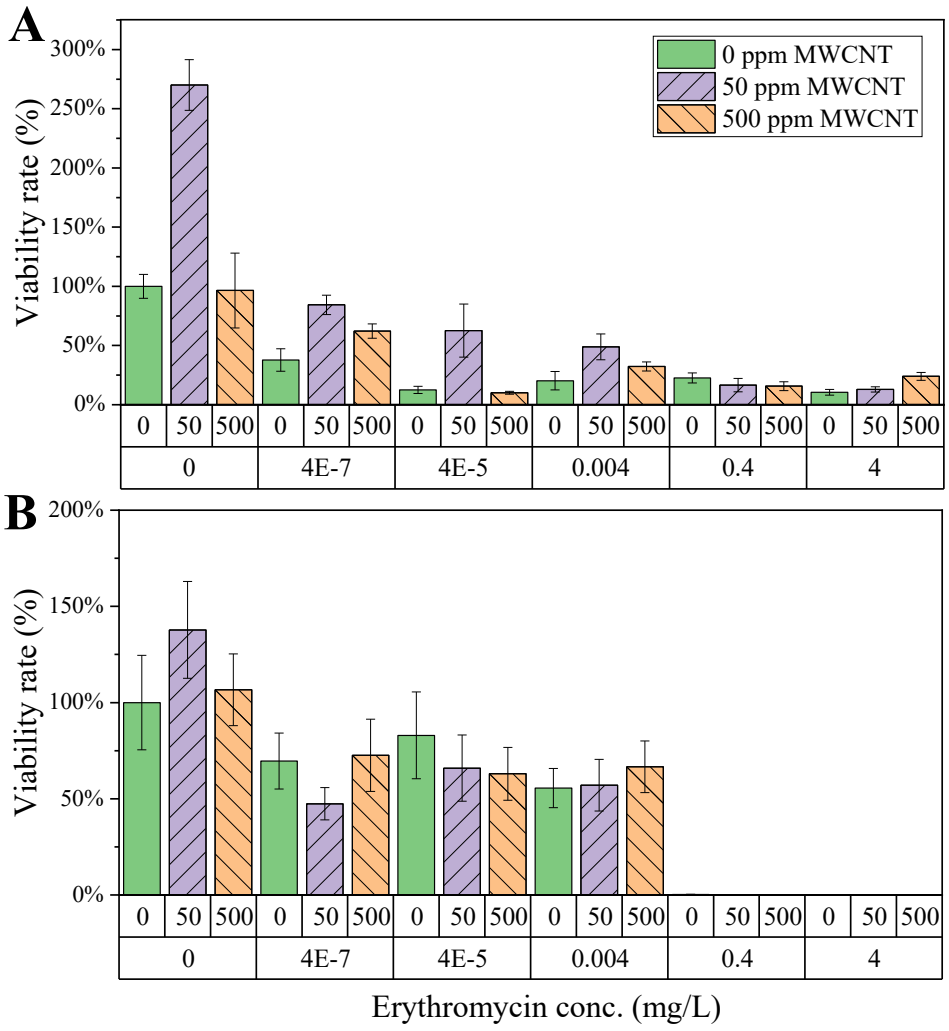

Figure 3-2 Effects of erythromycin and MWCNT on bacterial viability. (A) viability of *E. coli* (donor). (B) viability of *B. cereus* (recipient). Error bars represent the standard error of three replicates.

Many studies found that physiochemical properties of CNTs, such as the size and length of CNTs (Kang et al., 2008), impurity (the presence of heavy metal), and degree of dispersion with CNTs (Wick et al., 2007), were key factors on the cytotoxic to bacteria. The short length, disaggregated, and highly dispersed MWCNT have the highest bacterial toxicity, as it is easier for short length MWCNT to penetrate through cell membranes and show stronger antibacterial activity than longer length MWCNT (H. Chen et al., 2013). Another study reported that the viability of cells on the aggregated SWCNT was lower than that of the free-swimming cells, indicating the viability of free-swimming cells with SWCNT (from 1 to 50 μg/mL) was not concentration-dependent (Kang et al., 2007). Similarly, no growth delay and antibacterial acidity were observed against *Salmonella* and *Bacillus subtilis* cells with MWCNT range from 100 μg/mL to 500 μg/mL (Arias & Yang, 2006). The 100 μg/mL and 1000 μg/mL MWCNT with larger diameters (>50 nm) had more positive effects on the viability of telomerase-immortalized bronchiolar epithelial cells than 10 μg/mL MWCNT (Sohaebuddin et al., 2010). Conversely, the presence of 5 g/L CNT significantly enhanced (17 times) the methane production by *Methanobacterium formicicum* (Salvador et al., 2017). MWCNT is an effective adsorbent for removal various antibiotics and natural organic matter in aquatic environment (Hyung et al., 2007; Ji et al., 2009). The concentration of erythromycin was adsorbed by MWCNT to help bacteria to survive, and the adsorption rate of 50 ppm and 500 ppm MWCNT was 11.92% and 35.86%, respectively, with 60 mg/L erythromycin (R. Chen et al., 2018). Consequently, MWCNTs' toxicity varied on physiochemical properties of MWCNT and also depended on characteristics of bacteria and reaction systems (Sohaebuddin et al., 2010). From the viability results of this study, we concluded that the effect of erythromycin concentration was dominant and had a more significant impact than MWCNT in the combined effects of MWCNT and erythromycin on bacterial viability.

3.3.3 Morphology changes of bacterial cells by MWCNT treatment

In order to explore the effects of MWCNT against *E. coli* and *B. cereus*, SEM was provided to have a closer observation on the bacterial surface and evaluated the morphological changes with MWCNT treatment. After 4 hr incubation with 50 ppm and 500 ppm MWCNT, the observed *E. coli* and *B. cereus* cells were intact and no obvious damage on their outer membranes. The most noticeable effect of MWCNT was detected in donor strains, the surface walls of *E. coli* cells

incubated MWCNT (Figure 3-3) became more corrugated than that of *E. coli* incubated without MWCNT (Figure 2-4).

As shown in Figure 3-3 and Figure 3-4, MWCNTs had loose contact with donor and recipient cell walls by wrapping them around, which could protect the membranes from interacting with antibiotics. With higher concentrations of MWCNT, more surface areas of the cells were covered by MWCNT. Only a few open ends of nanotubes could be found in all SEM images. The open ends of nanotubes had larger probabilities to pierce into the cell wall like a needle, thus could potentially impair the cell wall (H. Chen et al., 2013; Kang et al., 2008). In this case, the cells mostly interacted with MWCNTs by their side walls and did not cause severe wall damages.

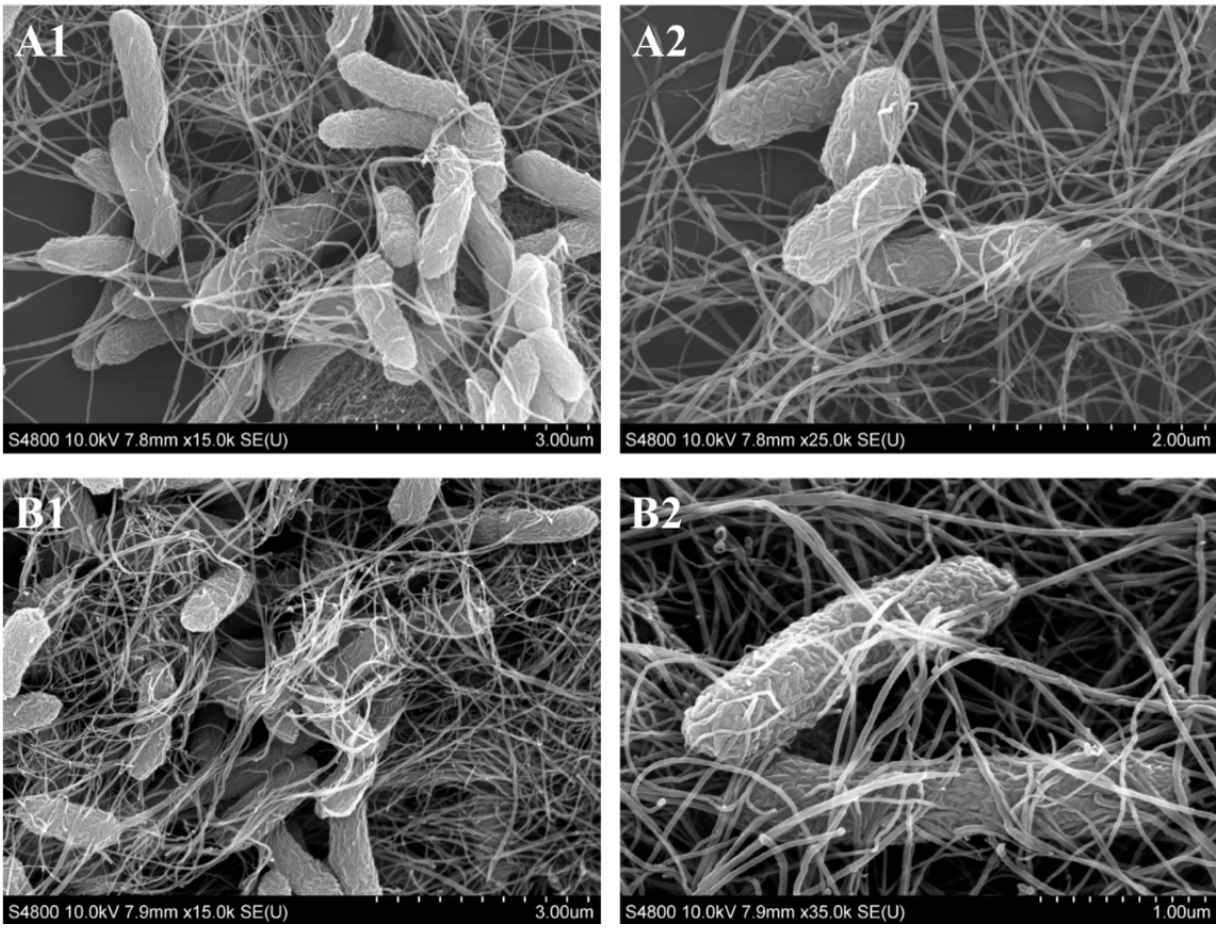

Figure 3-3 SEM images of *E. coli* after incubation with MWCNT suspension for 4 hr. (A1) and (A2) *E. coli* cells incubated with 50 ppm of MWCNT suspension. (B1) and (B2) *E. coli* cells incubated with 500 ppm of MWCNT suspension.

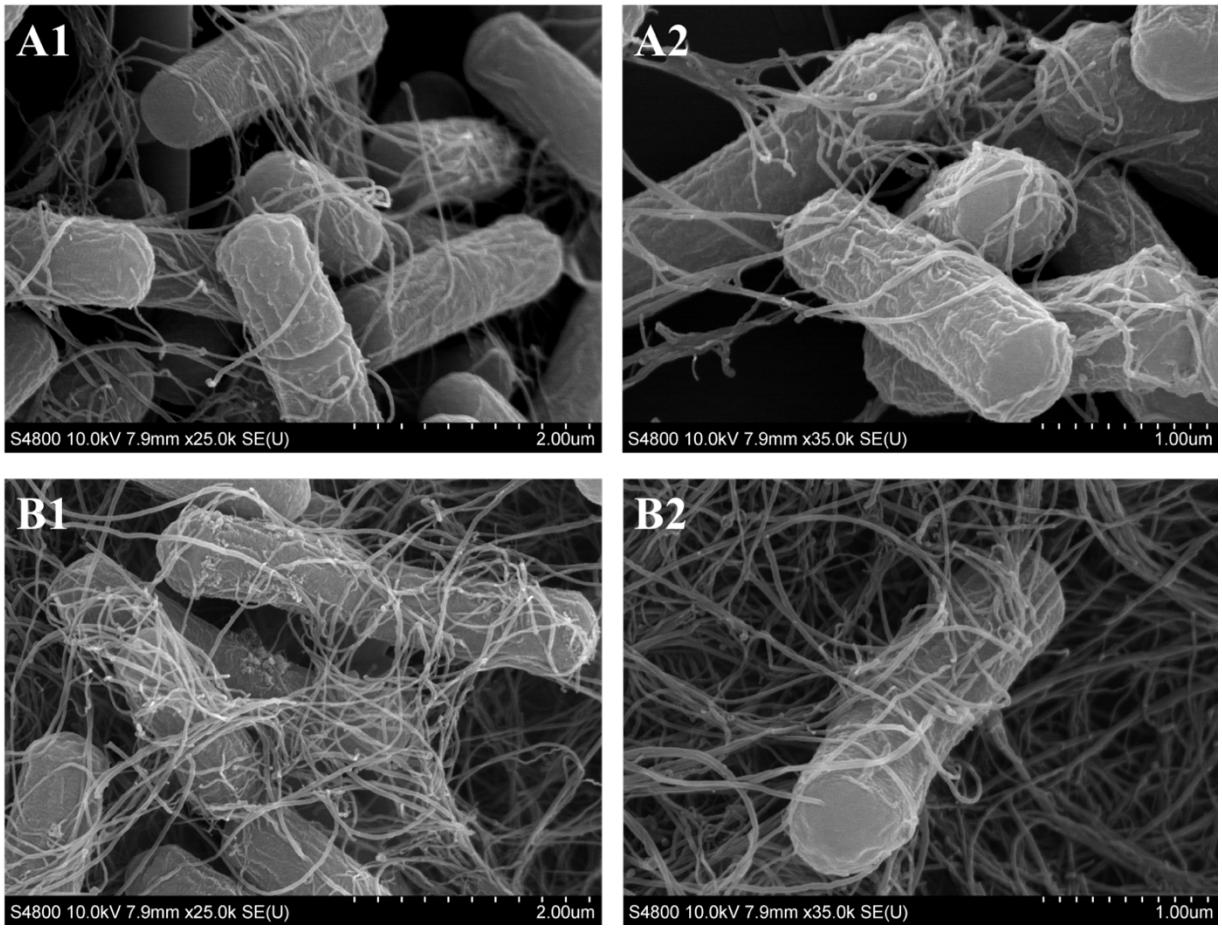

Figure 3-4 SEM images of *B. cereus* after incubation with MWCNT suspension for 12 hr. (A1) and (A2) *B. cereus* cells incubated with 50 ppm of MWCNT suspension. (B1) and (B2) *B. cereus* cells incubated with 500 ppm of MWCNT suspension.

It was likely that MWCNT provided protection for cell wall and thus increased the viability rate. Similar results were found in previous studies, in which bacteria were wrapped by graphene oxide nanosheets without external force and protected from external environment, resulting in the inhibition of HGT of *erm*80 genes (Akhavan et al., 2011; Hu et al., 2014; Zou et al., 2016). Additionally, cell walls of Gram-positive and Gram-negative bacteria are different. Cell walls in Gram-positive bacteria have a relatively thick layer (20-80 nm), and the cell walls of Gram-negative bacteria are thinner (10 nm) but has an outer membrane (Black, 2012). Thus, the average viability rate of *B. cereus* (Gram-positive bacteria) was higher than that of *E. coli* (Gram-negative bacteria).

Based on the length and diameter of bacteria measured on SEM images, donor and recipient cells exposed to different concentrations of MWCNT revealed morphological changes. The average length of *E. coli* after incubating with 50 ppm and 500 ppm MWCNT was longer than that of *E. coli* without MWCNT treatment, but the average diameter of *E. coli* exposed to MWCNT became smaller than that of *E. coli* in the control group Table 3-2. Therefore, the shapes (length/ diameter ratio) of donor strain were elongated due to the exposure of MWCNT. By contract, the average length of *B. cereus* after MWCNT treatment did not change much. The average diameter of *B. cereus* was increased from 0.70 to 0.85 and 0.87 μm, respectively, after incubating with MWCNT. Thus, the shapes of *B. cereus* were also changed but in the opposite way of *E. coli*.

Table 3-2 Morphology changes of *E. coli* and *B. cereus* treated with MWCNT.

	CNT conc. (ppm)	**0**	**50**	**500**
E. coli	Length (μm)	1.93 ± 0.026	2.02 ± 0.036	2.00 ± 0.038
	Diameter (μm)	0.73 ± 0.0069	0.62 ± 0.0038	0.63 ± 0.0038
	L/D ratio	2.64	3.24	3.19
B. cereus	Length (μm)	2.73 ± 0.14	2.69 ± 0.049	2.77 ± 0.049
	Diameter (μm)	0.70 ± 0.0076	0.85 ± 0.0039	0.87 ± 0.0047
	L/D ratio	3.89	3.17	3.18

3.3.4 Effects of erythromycin and MWCNT on the conjugation transfer

The effects of different concentrations of MWCNT on the transfer of *erm*80 gene were shown in Figure 3-5A. Addition of MWCNTs could inhibit the conjugative transfer compared with transfer efficiency in the absence of MWCNT. The numbers of transconjugants treated with 50 ppm MWCNT after 12 hr of incubation reached the lowest and were 1.73-fold lower than the numbers of transconjugants in control group (no MWCNT). At higher MWCNT concentration (500 ppm), the overall trends of transconjugants were slightly lower compared with the transconjugants' number in the absence of MWCNT. Due to the strong adsorption affinity of MWCNT, bacterial cells were able to attach on the MWCNT (Imaninezhad et al., 2018). MWCNT could cover around cells to prevent cells from accepting conjugative ARGs with external environment. It may explain the decreased numbers of transconjugants in the presence of MWCNT. When bacteria treated with MWCNT, the cells could be attached with MWCNT and reduce the cell to cell contact, thus decrease the conjugative transfer.

The combined effects of MWCNT and erythromycin on the conjugative transfer of *erm*80 gene were reported in Figure 3-5 B to F. The inhibited effect of MWCNT was still presented, and lower MWCNT concentration (50 ppm) inhibited the conjugative transfer more than higher concentration of MWCNT. Notably, almost no transconjugant was formed under 1 × MIC without MWCNTs, but with 50 ppm and 500 ppm MWCNT treatment, the numbers of transconjugants sharply increased to 660, and 9113, respectively, which also the highest transconjugants' numbers among all samples of mating. This result suggested that addition with MWCNT under lethal-level antibiotics could facilitate the transfer of ARGs between bacteria.

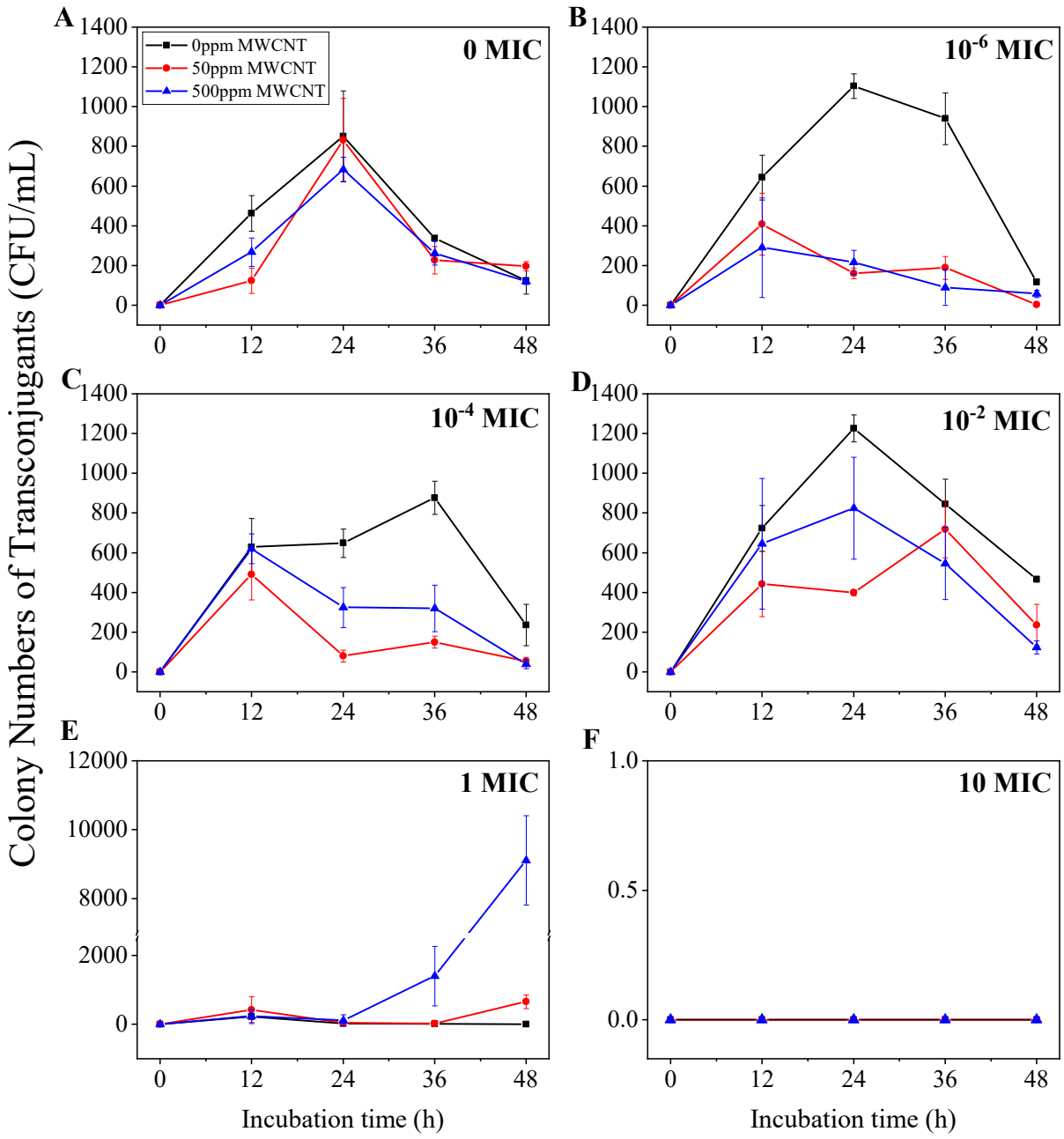

Figure 3-5 Effects of erythromycin and MWCNT on cultivable transconjugants. The concentrations of erythromycin exposure in panel A to F were 0, 4×10^{-7}, 4×10^{-5}, 4×10^{-3}, 0.4, 4 mg/L, respectively. Black squares: calculated transfer efficiency of *erm*80genes treated without MWCNT; red circles: calculated transfer efficiency of *erm*80genes treated with 50 ppm MWCNT; blue triangles: calculated transfer efficiency of *erm*80genes treated with 500 ppm MWCNT. Error bars represent the standard error of three replicates.

The results showed that transfer of *erm*80 resistant genes occurred under erythromycin and MWCNT (Figure 3-6). It was observed that the conjugative transfer frequency

(transconjugants/total numbers of recipients) was influenced by both concentrations of antibiotics and MWCNT. The combined effects of antibiotics and MWCNT tested in this study varied from synergistic to antagonistic with different concentrations of MWCNT and erythromycin. The effects of ARG transfer under both antibiotics and MWCNT at sub-MICs were inhibited, but the conjugative efficiencies were enhanced with MWCNT and lethal levels of erythromycin. The combination effect of 1× MIC of erythromycin and MWCNT was synergistic, and MWCNT in this mixture was able to lower concentration of erythromycin. The combination of sub-MIC concentrations of erythromycin and MWCNT weakened the transfer of *erm*80 elucidated that co-existence of MWCNT and erythromycin could change their individual effects on the conjugative transfer of *erm*80 genes, and the concentrations of MWCNT and antibiotics were significant for the spread of ARGs.

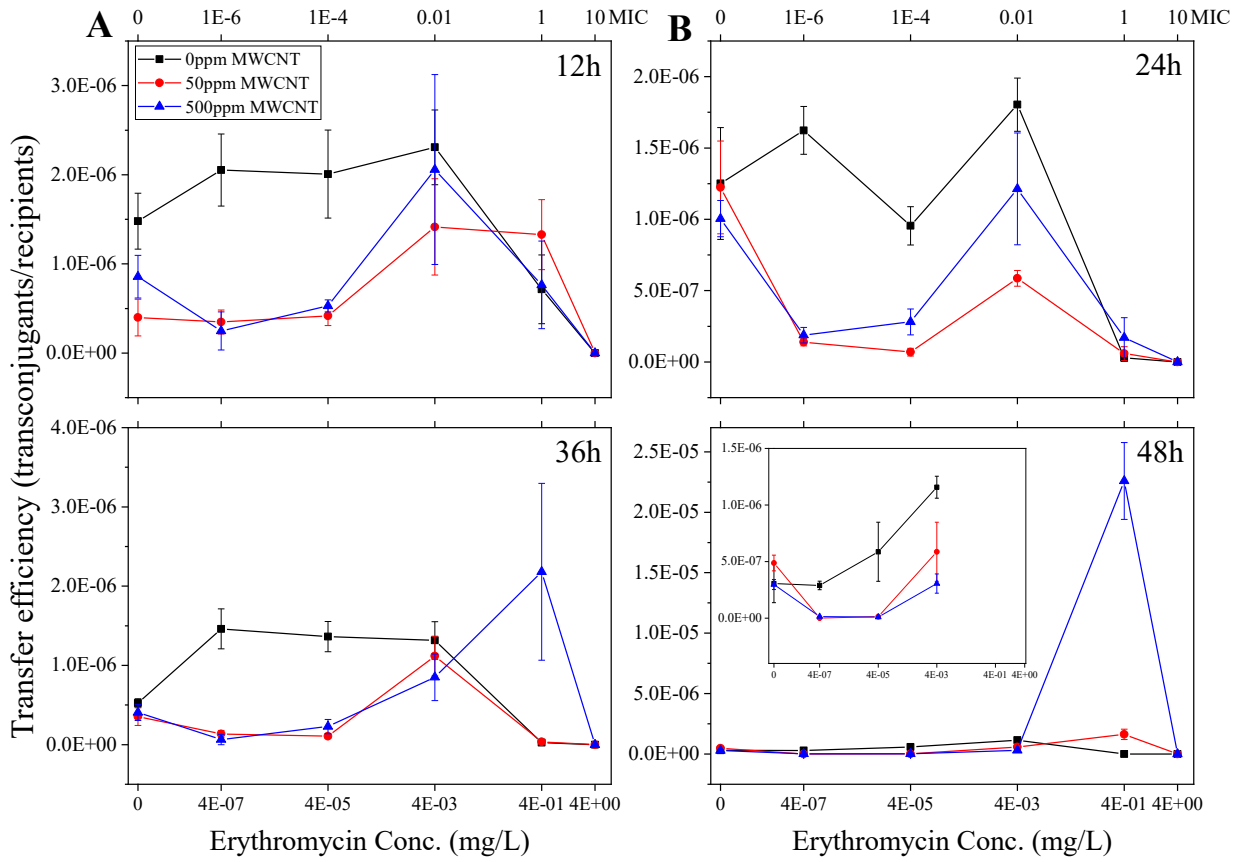

Figure 3-6 Effects of erythromycin and MWCNT on transfer efficiency. Black squares: calculated transfer efficiency of *erm*80genes treated without MWCNT; red circles: calculated transfer efficiency of *erm*80genes treated with 50 ppm MWCNT; blue triangles: calculated transfer efficiency of *erm*80genes treated with 500 ppm MWCNT. Error bars represent standard error of three replicates.

The combinations of sub-MICs of erythromycin and MWCNT resulted in either synergistic or antagonistic combination effects for the conjugation of *erm*80 genes between donor and recipient strains. There are two possible explanations for these results. First, conjugative transfer of ARGs is connected to the biofilm formation. It had been reported that MWCNTs had significant inhibition on biofilm formation and the lethal effects on biofilm formation increased with the length of MWCNT (Malek et al., 2016). The biofilm volumes of three opportunistic pathogens on 540 μm length MWCNT were significantly reduced to 2% and 13%, respectively. On the other hand, many existing studies have illustrated that conjugative transfer and biofilm formation are interconnected. Conjugative pili could promote cell-to-cell contact between donor and recipient cells which lead to biofilm formation (Ghigo, 2001), and the biofilm formation also could enhance

the conjugative transfer (Reisner et al., 2006). Therefore, inhibition on conjugative transfer of *erm*80 genes could be explained by the inhibitory effects of MWCNT on biofilm formation between donors and recipients. Addition with MWCNT in mating experiments decreased the biofilm formation between bacteria, thus reduced the transfer frequency of ARGs.

Additionally, the combination of mixture may result in stronger transfer of antibiotic resistance genes, when the mixture is more toxic than the individual compounds. But if the mixture is not toxic than the parent compounds, conjugative transfer would be weaker or unchanged. The high MIC concentrations of erythromycin could be adsorbed by MWCNT which make the erythromycin concentrations into non-lethal levels. Moreover, the non-lethal concentrations of erythromycin increased the growth of donors and recipients and enhanced the horizontal transfer between them. Therefore, the reactions between antibiotic and MWCNT could be influenced by their concentrations. The natural environments contain numerous pollutants and compounds, and the combination effects of these compounds could significantly affect the spread of antibiotic resistance genes, even though individual compound in environments is below the minimal selective concentration.

3.3.5 Abundance of 16S rRNA, *erm*80, and *intI*1 genes by real-time PCR

The changes of gene copy numbers of 16S rRNA in mating experiments with different concentrations of erythromycin and MWCNT were reported in Figure 3-7. The 16S rRNA concentrations were roughly stable (approximately 10^9 gene copies/mL) with sub-MIC erythromycin and MWCNT treatment, indicating that the gene copy numbers of 16s rRNA were not significantly affected by the sub-MIC concentration of erythromycin and MWCNT. Some fluctuations can be observed at higher concentrations of erythromycin. Specifically, the gene copies of 16S rRNA under 10 × MIC in the absence of MWCNT were $1.52 \pm 0.14 \times 10^8$ gene copies/mL which was one order of magnitude lower than samples with MWCNT at 12 hr. This result suggested that the addition with MWCNT could reduce the impact of lethal level of erythromycin, which is in accordance with previous results from culture-based experiments. Compared with the non-MWCNT control groups, the copy numbers of 16S rRNA genes did not changed significantly in MWCNT-exposure groups, indicating that the inhibition on *erm*80 genes and *intI*1 genes by MWCNT was not owing to the changes of the total microbial abundance.

With the increase of MWCNT concentration, the gene copy numbers of *erm*80 and *intI*1 genes were not significantly changed at 12 hr (Figure 3-8 and Figure 3-9). Under higher concentrations of erythromycin (1 × MIC and 10 × MIC), the abundance of *erm*80 and *intI*1 genes were increased with increasing concentrations of MWCNT. No similar results were observed for the abundance of 16s rRNA genes, indicating that the numbers of total bacteria were not related to the abundance of *erm*80 and *intI*1 genes, which could be explained by the fact that 16S rRNA genes are located in the chromosome, and the *erm*80 and *intI*1 genes are harbored by the pTE80 plasmid. Therefore, the damage of MWCNT for 16S rRNA, *erm*80, and *intI*1 genes were different, and the addition with MWCNT would influence on the plasmid not the chromosome. Moreover, *erm*80 and *intI*1 genes on the plasmid were affected with a significant correlation between them.

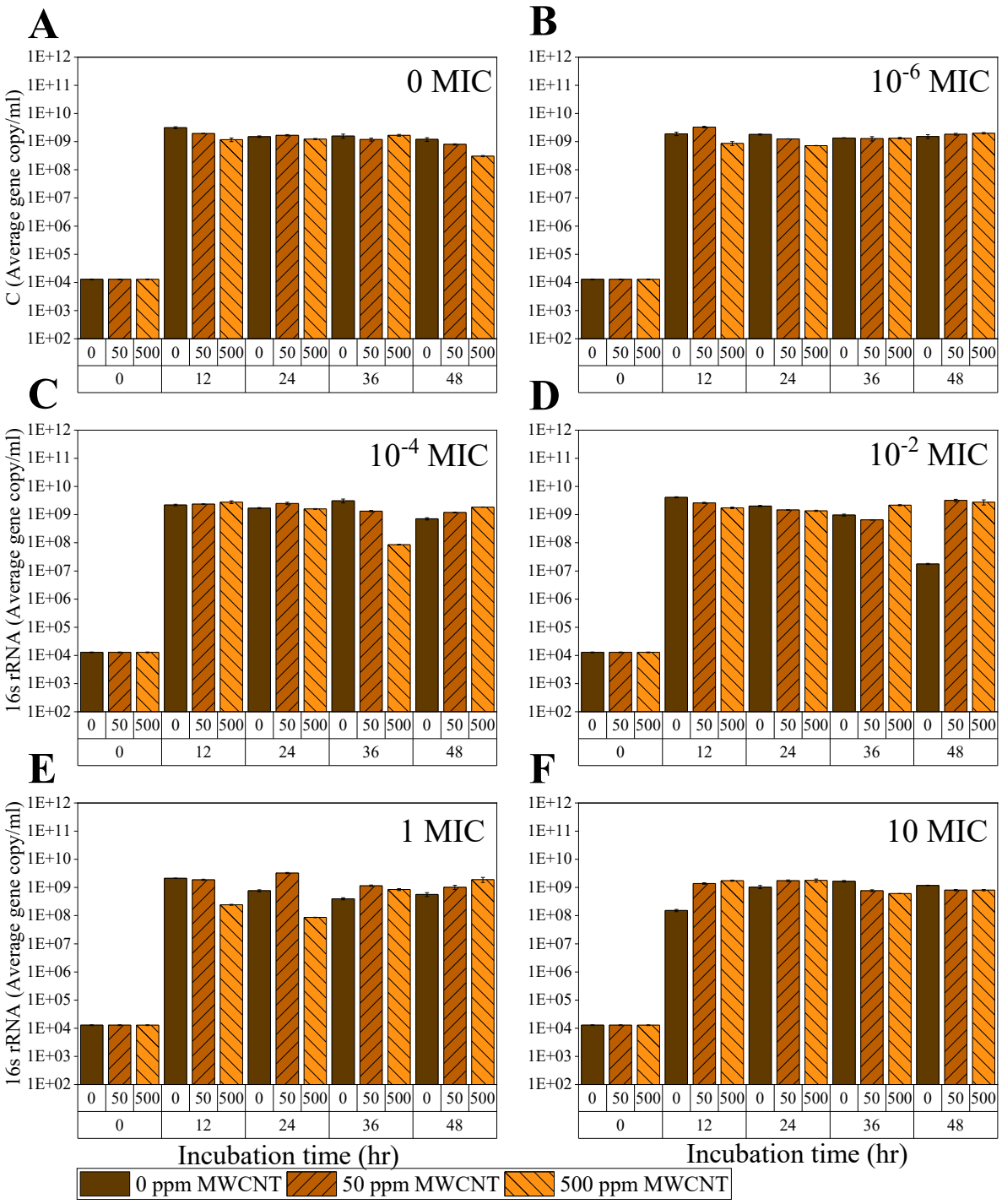

Figure 3-7 Effects of erythromycin and MWCNT on abundances of 16s rRNA gene. The concentrations of erythromycin exposure in panel A to F are 0, 4×10^{-7}, 4×10^{-5}, 4×10^{-3}, 0.4, 4 mg/L, respectively. Error bars represent the standard error of three replicates.

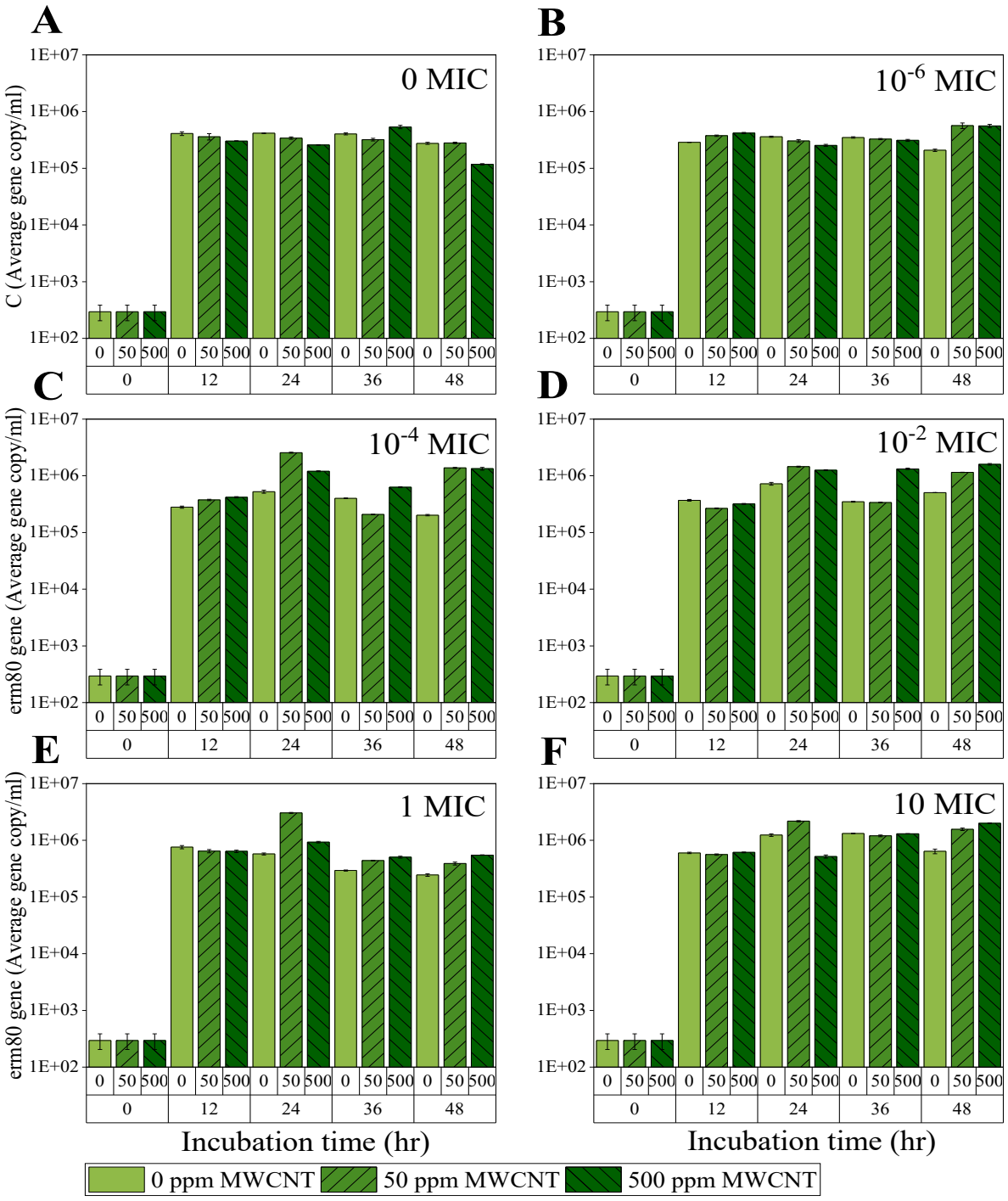

Figure 3-8 Effects of erythromycin and MWCNT on abundances of *erm*80. The concentrations of erythromycin exposure in panel A to F are 0, 4×10^{-7}, 4×10^{-5}, 4×10^{-3}, 0.4, 4 mg/L, respectively. Error bars represent the standard error of three replicates.

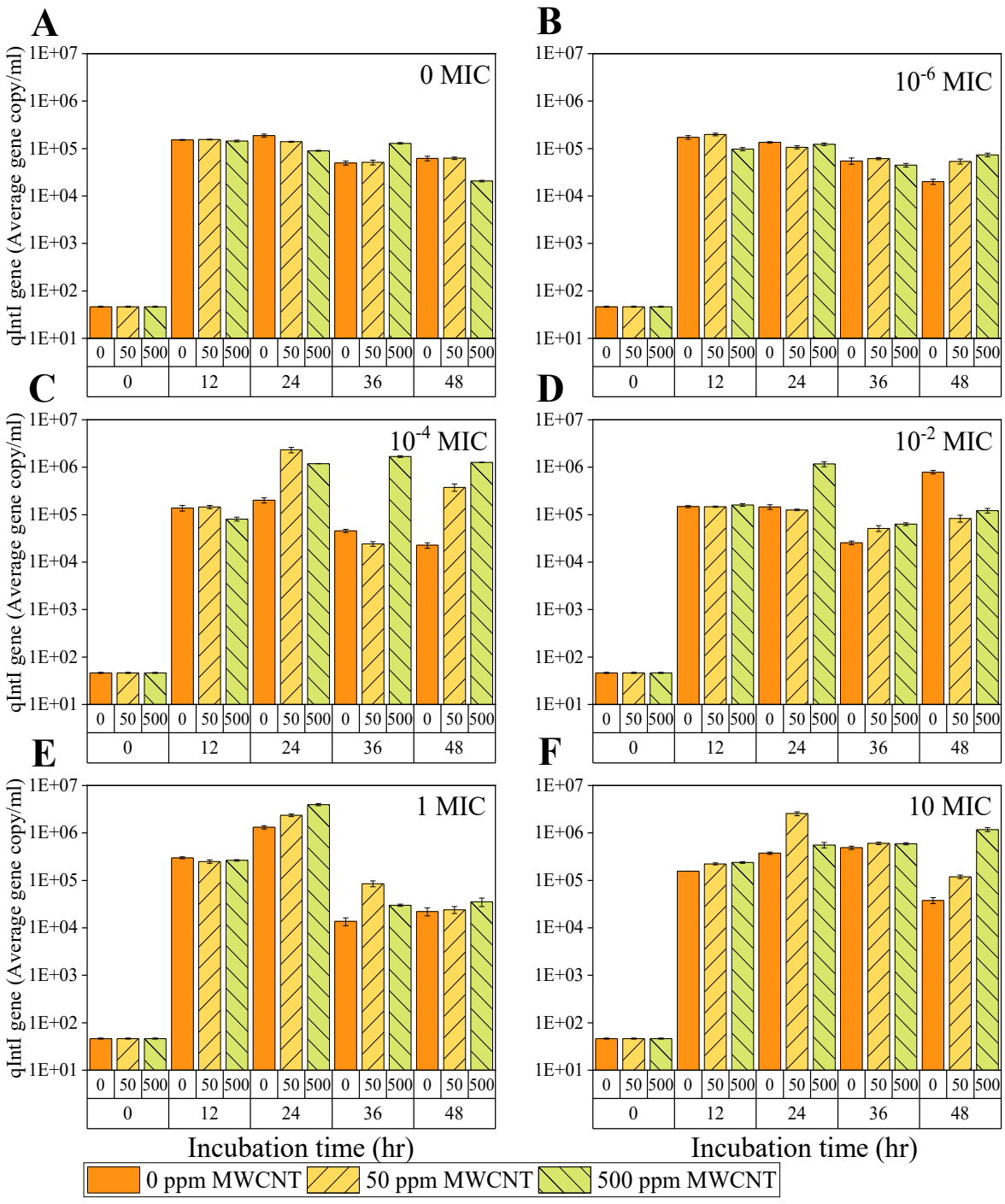

Figure 3-9 Effects of erythromycin and MWCNT on abundances *intI*1. The concentrations of erythromycin exposure in panel A to F are 0, 4×10^{-7}, 4×10^{-5}, 4×10^{-3}, 0.4, 4 mg/L, respectively. Error bars represent the standard error of three replicates.

3.3.6 Correlations between the abundance of *erm*80 and *intI*1 genes

The gene relative abundances of *erm*80 and *intI*1 genes with 50 ppm and 500 ppm MWCNT treatment were shown in Figure 3-10 and Figure 3-11. Although the bacterial viability was significantly affected by MWCNT, qPCR results were not significantly affected. The SOS response were not changed. The trends for relative abundance of *erm*80 genes with different concentrations of erythromycin and MWCNT followed a similar trend for the relative abundance of the *intI*1 genes. Significant correlations between *erm*80 genes and *intI*1 genes were observed, suggesting that the changes in *erm*80 and *intI*1 genes contribute to the induction of class 1 integrons. Sub-MIC antibiotics could induce *intI*1 genes and promote conjugative transfer of *erm*80 genes. In conclusion, induction of the SOS response by the combination of erythromycin and MWCNT can trigger the expression of *intI*1 genes, thus influencing the conjugative transfer of *erm*80 genes.

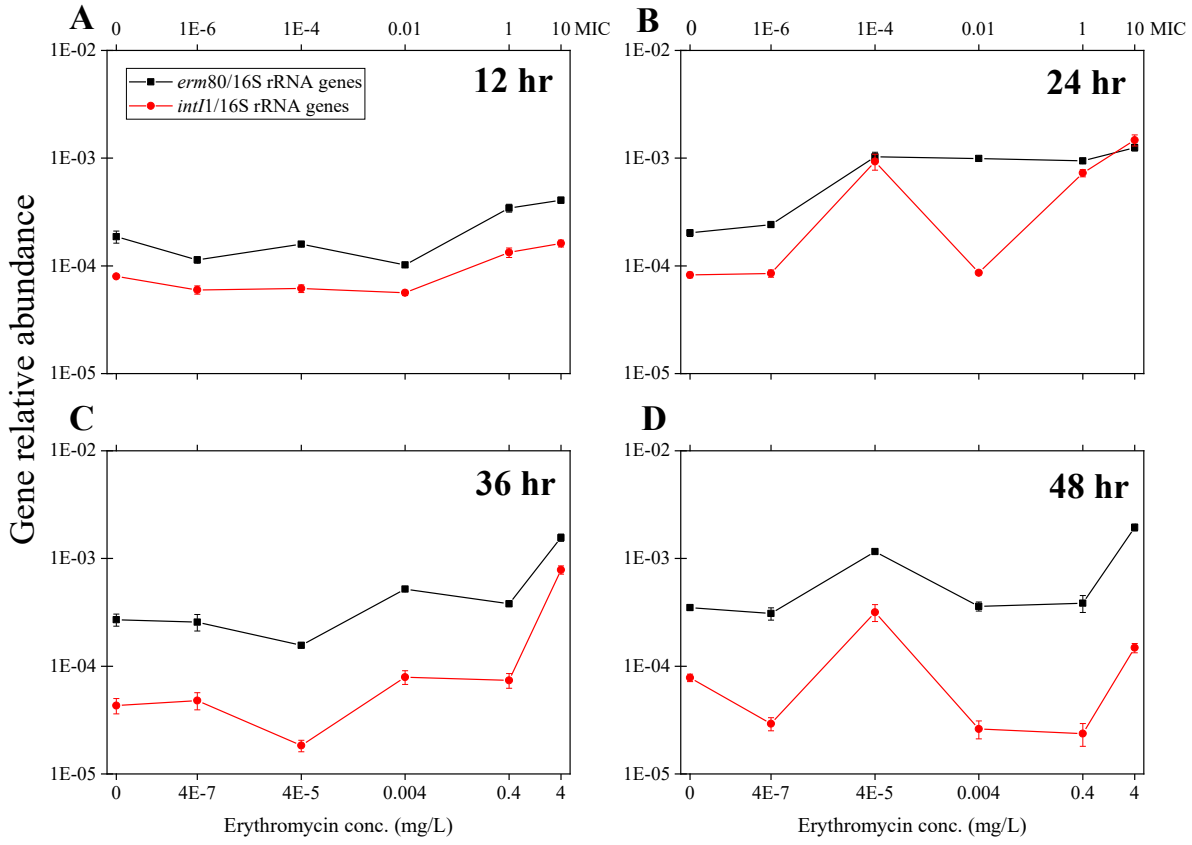

Figure 3-10 Effects of erythromycin and 50 ppm MWCNT on *erm*80 and *intI*1. Black squares: gene relative abundance of *erm80* genes; red circles: gene relative abundance of *intI*1 genes. Error bars represent standard error of three replicates.

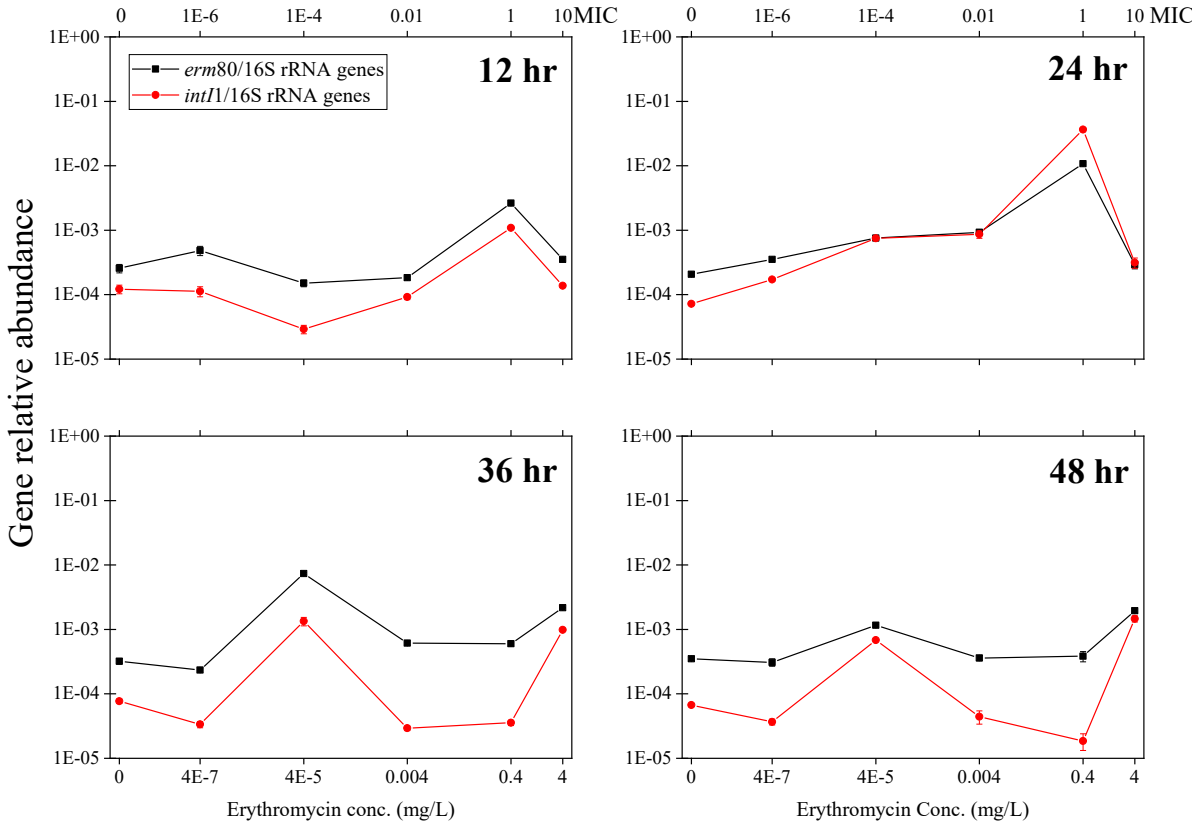

Figure 3-11 Effects of erythromycin and 500 ppm MWCNT on *erm*80 and *intI*1. Black squares: gene relative abundance of *erm80* genes; red circles: gene relative abundance of *intI*1 genes. Error bars represent standard error of three replicates.

3.3.7 Cell membrane integrity analysis

3.3.7.1 OD_{260nm} results

Leakage of intracellular DNA and RNA is an indicator of bacterial membrane integrity, as the cell membrane could be damaged during exposure to MWCNT (H. Chen et al., 2013). When the cell membrane become compromised with MWCNT treatment, intracellular contents such as DNA and RNA would be released from cells. The OD_{260nm} results (Figure 3-12A) showed that the amount of intracellular DNA and RNA decreased with increasing concentrations of MWCNT.

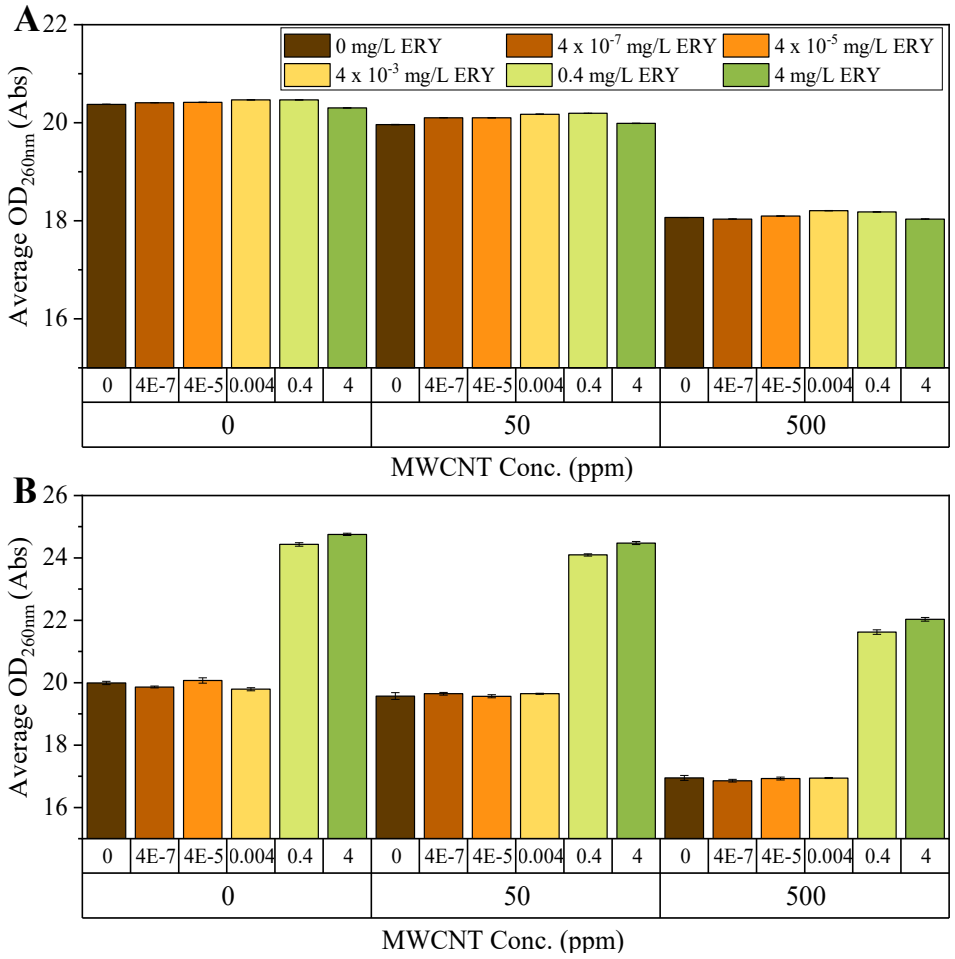

Figure 3-12 OD_{260nm} results of (A) donor and (B) recipient. Error bars represent the standard error of three replicates.

When the MWCNT concentration rise to 500 ppm, the OD_{260nm} values of *E. coli* reduced by 1.13 times compared to the values of *E. coli* without MWCNT treatment. Similar results were also observed on the recipient cells, except under lethal level of erythromycin. This is a direct evidence confirming that the HGT rates decreased with the addition of MWCNT, as the intracellular ARGs decreased due to smaller amounts of damaged donors. The results are also consistent with previous observation that MWCNT also increased the viability for both donors and recipients.

Overall the effect of erythromycin on cell membrane integrity was dominant in the combination of erythromycin and MWCNT on bacterial cells, especially on *B. cereus*. As shown in Figure 3-12B, the higher concentrations of erythromycin significantly increased the OD_{260nm} values of *B. cereus*. Lethal concentration of erythromycin could inhibit or even kill the growth of *B. cereus*,

thus more intracellular DNA and RNA would be release from impaired cells. The membrane integrity results of recipient were consistent with previous viability with erythromycin treatment., indicating that the decreased viability rate may be associated with the impaired membrane of cells. It is interesting that the OD_{260nm} results were not in accordance with the viability rate of *E. coli*, but were consistent with the transfer frequency of *erm*80 genes in mating experiments. This result may suggest that the membrane integrity of *E. coli* is a key factor to influence conjugative transfer.

3.3.7.2 Cell membrane permeability

On the basis of the mating experiments and OD_{260nm} tests, we conclude that the compromised membrane of *E. coli* cells treated with MWCNT likely resulted in the horizontal transfer of *erm*80 genes. Thus, fluorescence-based assays were conducted for *E. coli* cells. Treated donor cells stained with SYBR Green I and PI dyes display that many of them were alive and contained intact membranes after 4 hr incubation with different concentrations of MWCNT. As shown in Figure 3-13 and Table 3-3, cell membrane permeability of donors with exposure of 500 ppm MWCNT was significantly increased up to approximately 53-fold higher than untreated groups (1%).

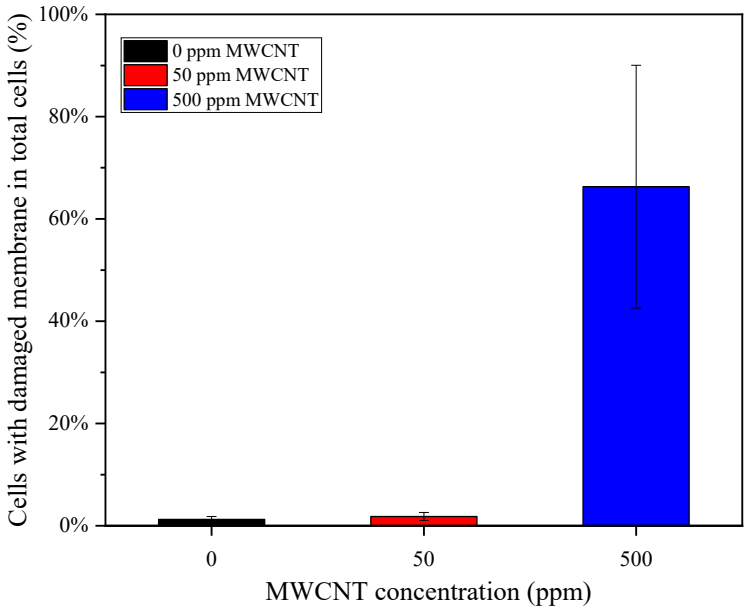

Figure 3-13 Effects of MWCNT on cell membrane permeability. Black: percentage of cells with damaged membrane in total cells treated with 0 ppm MWCNT suspension; red: percentage of cells with damaged membrane in total cells treated with 50 ppm MWCNT suspension; blue: percentage of cells with damaged membrane in total cells treated with 500 ppm MWCNT suspension. Error bars represent standard error of three replicates.

Table 3-3 Cell membrane permeability

Cell type	Erythromycin conc. (mg/L)	CNT conc. (ppm)	Intact membrane cells# (cells/mL)	Impaired membrane cells# (cells/mL)
E. coli	0	0	$4.51 \pm 0.60 \times 10^9$	$5.67 \pm 2.40 \times 10^7$
E. coli	0	50	$3.77 \pm 0.77 \times 10^9$	$6.83 \pm 2.58 \times 10^7$
E. coli	0	500	$2.47 \pm 0.57 \times 10^9$	$1.64 \pm 0.45 \times 10^8$

The percentage of cells with damaged membrane enhanced with increasing concentrations of MWCNT (from 0 to 500 ppm), resulting in the release of cytoplasmic materials from the cells into solutions. More intracellular DNA from *E. coli* released to extracellular environment would lead to higher transfer frequency of *erm*80 genes.

The number of cells detected by fluorescence microscopy was comparable with culturable colony numbers and 16S rRNA gene copy numbers in this study. However, the viability rates of *E. coli* with MWCNT treatment were different from the results of cell membrane integrity. The interaction among bacterial cells, MWCNT, and erythromycin is still unclear. We speculate that although the MWCNT damaged the cell membrane, the cells with more permeable membrane were not be killed but entered the VBNC state. Consequently, the bacterial viability would not be significantly impacted by the damaged cell membrane. In addition to the physical damage on the cell membrane with wrapping around the cell, the MWCNT also adsorbed the erythromycin and DNA in mating experiments (R. Chen & Zhou, 2018; Zhao & Johnson, 2007).

3.4 Conclusion

MWCNTs have been studied in various fields, including biology, chemistry, and environmental treatment. However, little is known about the individual effects of MWCNT and combined effects of MWCNT and antibiotics on the development and proliferation of ARGs. This is the first study to investigate the effects of antibiotics and MWCNT on the horizontal transfer of ARGs at both low and high concentrations. Under sub-MIC levels of erythromycin, the addition with MWCNT could inhibit conjugative transfer of *erm*80 genes between *E. coli* and pathogen *B. cereus*. But the MWCNT promoted the horizontal gene transfer above 1 × MIC, and the mating experiments

showed that the transfer frequency was up to 101-fold higher in the 1 × MIC of erythromycin with MWCNT treatment than that in the no MWCNT treatment groups. The transfer of antibiotic resistance plasmids could be influenced by the concentrations of antibiotics and MWCNTs.

Although the concentration of individual stressor is not enough to confer antibiotic selection, the effects of the antibiotic resistance to both antibiotics and MWCNTs above 1 ×MIC could add up and help to select for antibiotic resistance. The MWCNT significantly decreased the transfer frequency of *erm*80 by 1-2 orders of magnitude compared with control groups. These results suggest that MWCNT could be applied to control the occurrence and spread of ARGs and to mitigate the selective pressure of antibiotics on horizontal transfer of ARGs.

CHAPTER 4. REMOVAL OF BACTERIAL DNA AND ANTIBIOTIC RESISTANCE GENES BY ELECTROCHEMICAL MWCNT FILTRATION

4.1 Introduction

Extracellular, or naked DNA, represents the DNA located outside of the cell and can be found in soils, sediments, and natural waters (Nagler et al., 2018). Naked DNA was mostly studied for its ability of transformation, and antibiotic-sensitive microorganism could uptake naked DNA with ARGs from their surroundings (Levy et al., 2004). Unlike the processes of conjugation and transduction, transformation does not need donor bacteria or viruses to transfer their ARGs to the recipients. Therefore, it is crucial to control the dissemination and prevalence of extracellular DNA, especially those carrying ARGs. Different forms of ARGs, including bacteria and viruses carried with ARGs, and naked DNA with ARGs were mainly from WWTP effluent (Kümmerer, 2009) and its effluent receiving river (Xu et al., 2015). Although wastewater treatment processes could kill or inactivate the bacteria with ARGs, they are not designed to remove the DNA in wastewater. ARGs still have potentials to transfer downstream and to environments. To address the threats of antibiotic resistance and ARGs, DNA removal before discharging or other applications is critical.

In natural environments, biological, physical, and chemical factors such as pH, ionic concentrations of solute, and NOM have impacted the fate of extracellular DNA (Levy-Booth et al., 2007). The surface charge and structure of DNA could be changed because of folding and supercoiling under a variety of environmental factors. Several studies have investigated (Lu et al., 2012; Nguyen et al., 2007) the adsorption mechanisms of DNA interacting with NOM-coated silica surface, and their effects on horizontal gene transfer through natural transformation. The obtained results suggested that DNA adsorption was likely from divalent cation complexation with the phosphate backbone of DNA. Additionally, another study has demonstrated that the removal of ARGs could be promoted by native wastewater colloids in membrane filtration due to colloidal interaction with DNA (Breazeal et al., 2013). NOM is naturally originated from environments and can be widely found in the subsurface environment (Nguyen & Elimelech, 2007). Previous study has demonstrated that NOMs have a significant impact on the persistence of DNA and ARGs, indicating that the complexes of humic acid and DNA still retain the capability of ARGs

transformation (Crecchio et al., 2005).Therefore, it is important to evaluate the effect of NOM and other environmental factors on the performance of removing extracellular DNA and ARGs.

The objective of this study was to remove both extracellular DNA and ARGs efficiently in a wide range of conditions, including both natural environments and contaminated water systems with a robust system and low operating cost. We chose MWCNT filters and electrochemical filtration method due to their low cost and simple setup (H. Liu et al., 2014; Y. Liu et al., 2015). To evaluate the performance, we investigated the removal efficiency of bacterial DNA and ARGs by electrochemical MWCNT filtration, quantified the performance of MWCNT filter in removing bacterial DNA and ARGs with a variety of environmentally relevant factors in the solution, and estimated the filtration performance of DNA and ARGs removal under the effect of NOM.

4.2 Materials and Methods

4.2.1 Characterization of electrochemical MWCNT membrane and electrolyte

High conductivity MWCNT blend buckypaper with an average weight of 45 GSM was purchased from Nanotechlabs (Yadkinville, NC) and trimmed to circular membranes (diameter: 42 mm) for electrochemical filtration. The average thickness of the MWCNT is 15 to 250 micron and the purity is 95 % according to the manufacturer.

Sodium sulfate is commonly found in aquatic systems and also normally used as background electrolyte for electrochemical water filtration. Therefore, Na_2SO_4 was selected as the background electrolyte in this study. 1M electrolyte stock solution was prepared by dissolving 71.018 g ACS reagent Na_2SO_4 (Sigma-Aldrich, St. Louis, MO) in nanopure water. All electrochemical filtration experiments were performed at 0.1 M Na_2SO_4 unless noted otherwise. The Na_2SO_4 electrolyte solution was autoclaved at 121°C before each experiment in order to ensure its sterility.

4.2.2 Bacteria and genomic DNA preparation

E. coli bacteria with *erm*80 resistant gene was isolated from Wabash River, and used to prepare DNA and ARGs for the filtration experiments. Host *E. coli* cells were grown in LB broth containing 50 mg/L erythromycin with 180 rpm shaking at 37 °C until they reached stationary growth phase (12-16 hr incubation) before use.

The DNA extraction method from (Russell & Sambrook, 2000) was used in this study. 10 ml of cell suspension were harvested by centrifugation at 4000 g for 10 min until a compact pellet formed. To lyse the bacterial cell walls, the pellet was resuspended in a mix of 2.835 mL of Tris-EDTA buffer, 0.15 mL of 10% SDS, and 0.015 mL of 20 mg/mL proteinase K solution, and incubated for 1 hr at 37 °C. 0.5 mL of 5 M NaCl and 0.04 mL of CTAB/NaCl solution were then added to the lysate for 10 min at 65 °C. Equal volume of chloroform/isoamyl alcohol (24/1 vol/vol) was added and spun for 5 min in centrifuge to remove the CTAB-protein/polysaccharide complexes. The aqueous supernatant was removed to a fresh centrifuge tube and added with an equal volume of phenol/chloroform/isoamyl alcohol (25/24/1 vol/vol/vol) to purify the extracted DNA. After 5 min centrifugation, the supernatant was transferred to a fresh tube and added with 0.6 vol isopropanol to precipitate the DNA. A white stringy DNA pellet was formed after shaking and transferred to 70 % ethanol solution, followed by 5min centrifugation to wash off the residual chemicals. The supernatant was discarded, and the DNA pellet was air-dried in a biosafety cabinet overnight before resuspended in 1 mL of sterile nanopure water. The concentrations and purities of DNA were quantified with a NanoDrop 2000c UV-Vis Spectrophotometer for further use.

4.2.3 Electrochemical filtration reactor and experiments

The cyclic voltammetry (CV) and breakthrough curve analyses were conducted on a CHI 604E electrochemical analyzer (CH instruments, Austin, TX) with three electrodes: a MWCNT anodic filter connected to the titanium anodic ring as the working electrode, a titanium counter electrode connected to the perforated titanium shim disk, and a silver/silver chloride reference electrode. A schematic of the MWCNT electrochemical filtration reactor is presented in Figure 4-1.

The CV experiment was an electrode technique used in this study to investigate the applied potential range of reduction and oxidation processes. The applied potential range was from -3V to 3V and the scan rate was 0.1 V/s. The reference electrode was placed at the outlet of the reactor to connect the effluent aliquots.

In the electrochemical filtration experiments, 0.1 M Na_2SO_4 solution was first pumped through the filtration reactor at a flow rate of 1.5 mL/min using a peristaltic pump for 30 min without an applied

voltage. After the priming process, a specific electric potential was applied to the reactor via the electrochemical analyzer. The DNA suspensions with 0.1 M Na_2SO_4 was then flowed through the MWCNT filter at 1.5 mL/min, and the effluent samples were directly collected from the filter outlet in sterilized centrifuge tubes at each collection time for 50 min.

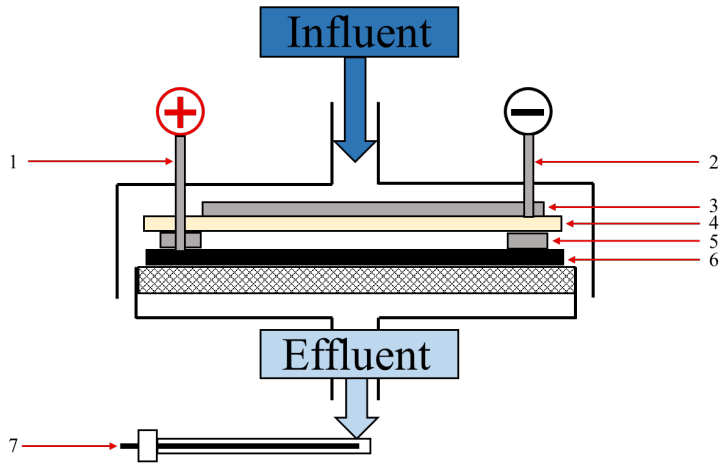

Figure 4-1 MWCNT electrochemical filtration apparatus (Y. Liu et al., 2015). (1) a titanium anode. (2) a titanium cathode. (3) a perforated titanium shim disk. (4) an insulated silicone ring. (5) a titanium anodic ring. (6) a MWCNT anodic filter. (7) a Ag/AgCl reference electrode connected to an electrochemical analyzer. The elements from (1) to (6) were contained in a modified swin-lock plastic filter holder (Whatman, Maidstone, UK).

4.2.4 Impact factors on electrochemical MWCNT filtration

It is important to evaluate the performance of MWCNT filtration at different conditions, and selected factors were applied potential, pH, phosphate, and NOM. All chemicals used in this study were analytical grade.

A range of applied potentials was conducted by the electrochemical analyzer to evaluate the most efficient voltage for the electrochemical removal of DNA and ARGs. The applied potentials used in this study were 0 V, 0.25 V, 0.5 V, 0.75 V, 1 V, and 2 V.

For pH experiments, the primary purpose was to investigate the performance of electrochemical MWCNT filtration in a wide range of pH. The pH of sample solution was adjusted by 0.1 M NaOH

or HCl, to 5, 7, 9, 11 and 13 for each experiment. An Orion versa star pro multiparameter benchtop meter with pH probe (Fisher Scientific, Hampton, NH) was utilized to measure pH.

The aim for adding phosphate ions in the DNA solution was to examine the interaction between sugar phosphate backbone of DNA and the anodic MWCNT filters. Addition of 0 M, 0.0313 M, 0.0625 M, and 0.125 M of Na_2HPO_4 with 0.1 M Na_2SO_4-DNA solution were tested. 0.5 M of Na_2HPO_4 stock solution was prepared by dissolving Na_2HPO_4 (Sigma-Aldrich, St. Louis, MO) to nanopure water. The desired concentrations of working solution were prepared by diluting the stock solution. The working solutions of Na_2HPO_4 were autoclaved before each use.

A commercial humic acid (Sigma-Aldrich, St. Louis, MO) was employed as a model natural organic matter in aquatic systems. A humic acid stock solution was prepared by dissolving 100 mg of humic acid powder in 200 mL of nanopure water, followed by stirring for about 15 hr in the dark and adjusting pH to 8 by 0.1 M NaOH. The stock solution was sonicated for 15 min with the amplitude at 20% by a sonic dismembrator (Fisher Scientific, Hampton, NH), and then vacuum filtered by a 0.22 μm mixed cellulose esters membrane (MilliporeSigma, Burlington, MA). The desired concentrations of NOM solution were prepared by diluting the 500-ppm stock solution. The electrochemical filtration experiments were completed with four concentrations of NOM solution (0 ppm, 5 ppm, 10 ppm, and 20 ppm).

4.2.5 Measurement of the nucleic acid concentration by UV-Vis spectrophotometer

To measure DNA concentrations, absorbance method was applied with a NanoDrop 2000c UV-Vis Spectrophotometer. DNA absorbs UV light at 260 nm the strongest, and the absorbance at 260 nm allows us to calculate the concentration of DNA samples based on the following modified Beer-Lambert equation according to Nanodrop 2000c manual (Scientific, 2009). The detection range is from 2 ng/μL to 15,000 ng/μL for dsDNA. All tests were performed in triplicates. DNA concentration was calculated with the of $c = A \times \varepsilon / b$, where c is the DNA concentration in ng/μL, A is the absorbance reading at 260 nm, ε is the extinction coefficients for double-stranded DNA which is 50 ng-cm/μL, and b is the pathlength in cm.

4.2.6 Quantification of the bacterial DNA genes and ARGs by real-time PCR

16S rRNA genes and *erm*80 genes were quantified by real-time PCR. The qPCR experiments were conducted on a CFX96 Real-time PCR detection system (Bio-rad, Hercules, CA).

4.2.7 Scanning Electron Microcopy for MWCNT filters

To observe the surface morphology of the MWCNT filters and the effects of electrochemistry on the DNA on the MWCNT filters, SEM was employed in this study. The MWCNT filters before and after filtration with DNA solution were immediately collected and air dried in the biosafety cabinet to protect from the free DNA in the air. The samples were imaged using a FEI Quanta 3D FEG dual-beam SEM.

4.3 Results and Discussion

4.3.1 Cyclic voltammetry curve of MWCNT filters

CV measurements on the presence and absence of bacterial DNA in 0.1 M Na_2SO_4 solution were conducted to identify the favorable range of applied potentials for electrochemical filtration experiments (Figure 4-2).

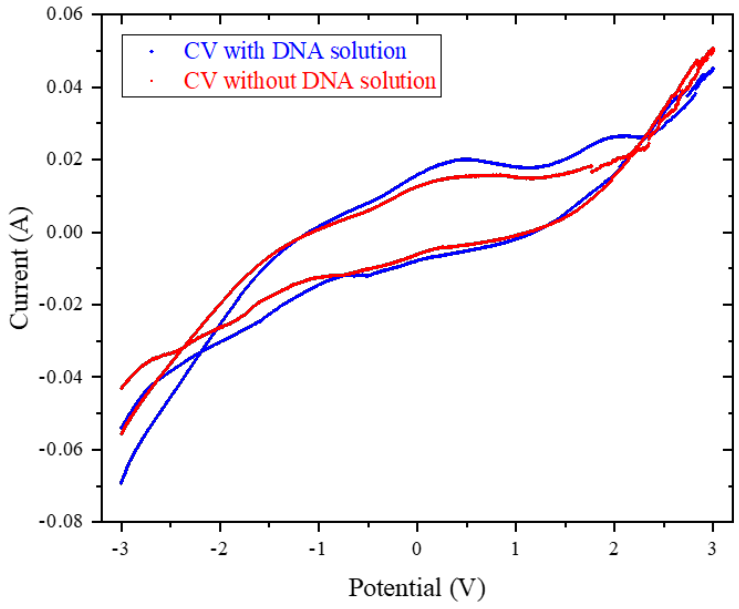

Figure 4-2 CV curves of DNA solution and control. CV curve of DNA sample with 0.1 M Na_2SO_4 as background electrolyte (red line); CV curve of 0.1 M Na_2SO_4 (blue line).

The two peaks (at +0.5 V and at +2 V) were displayed in the oxidation process, and a reduction peak at -0.52 V. Meanwhile, no obvious peaks were observed in the control group. The backbone phosphates of DNA are responsible for the natural state of double stranded DNA to appear negatively charged. The acidic phosphate and sugar molecules in the backbone of DNA shield the nitrogenous bases on the outside. Therefore, the negative charges of DNA are more likely to attract an anodic MWCNT membrane than a cathodic MWCNT membrane. These results help us to set the appropriate range of voltage from 0 V to 2 V.

4.3.2 SEM images of MWCNT filters

SEM was used to examine the surface morphology of the MWCNT membrane before and after DNA filtration. As shown in the SEM images (Figure 4-3 A1&A2), the distribution of MWCNT was uniform.

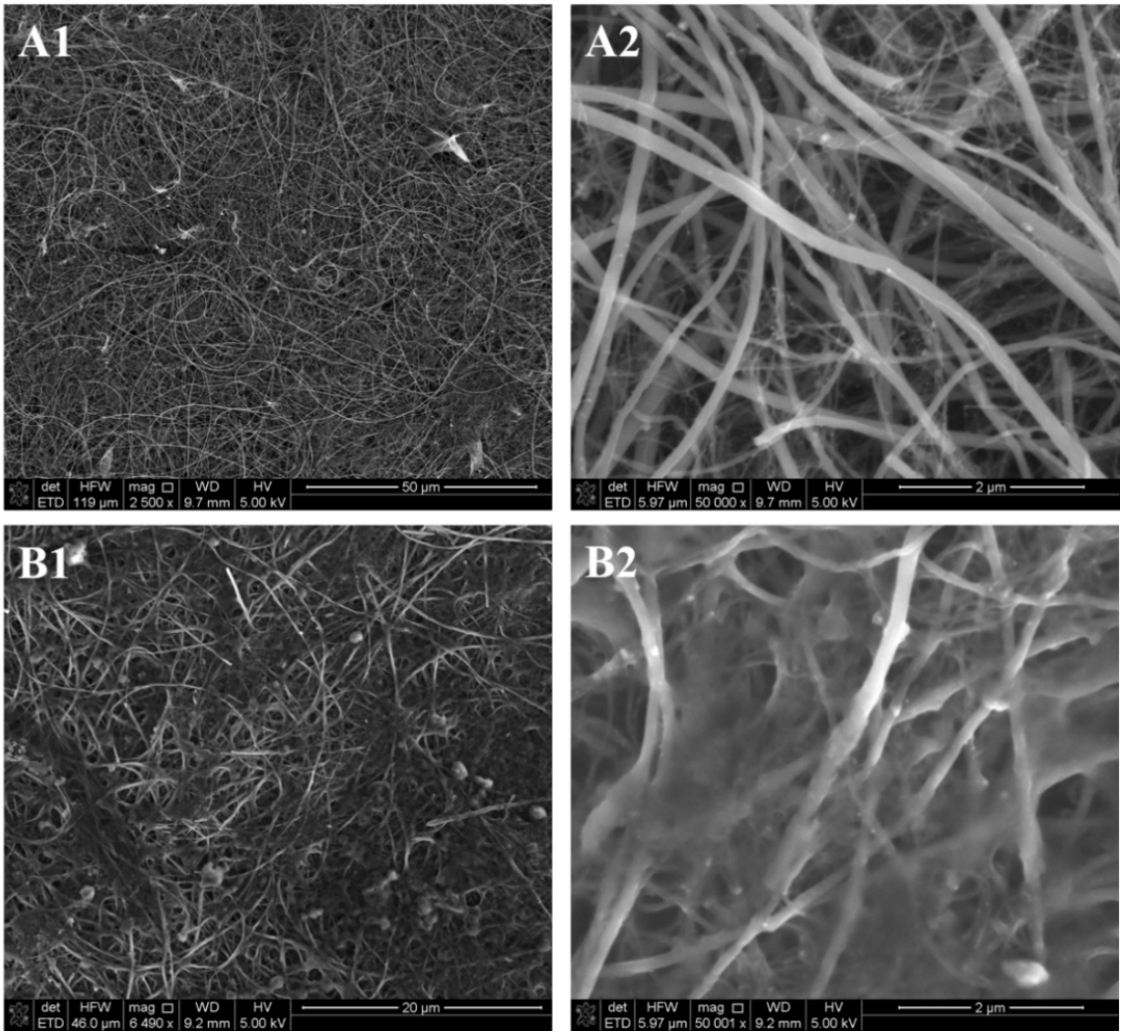

Figure 4-3 SEM images of DNA on MWCNT before and after electrochemical filtration. (A1) MWCNT filter before filtration experiments under 2,500 x magnification. (A2) MWCNT filter before filtration experiments under 15,000 x magnification. (B1) genomic DNA retained on MWCNT filter. (B2) genomic DNA retained on MWCNT filter.

After electrochemical filtration with DNA solution, the morphology on the surface of MWCNT membrane was different, and bacterial DNA attached to the MWCNT filter can be obviously observed (Figure 4-3 B1&B2). The SEM images indicated that genomic DNA were retained on the MWCNT filter after electrochemical filtration.

4.3.3 DNA and ARGs removal under different solution chemistries.

4.3.3.1 Effects of applied potential

To find the most efficient applied potential for electrochemical bacterial DNA filtration, 0 V, 0.25 V, 0.5 V, 0.75 V, 1 V, and 2 V were selected based on the CV curve. There was a general upward trend of DNA removal efficiency with increasing electric potentials, until saturated around 0.75 V. The breakthrough curves for DNA solution onto the MWCNT membrane were shown in Figure 4-4, Figure 4-6, and Figure 4-8.

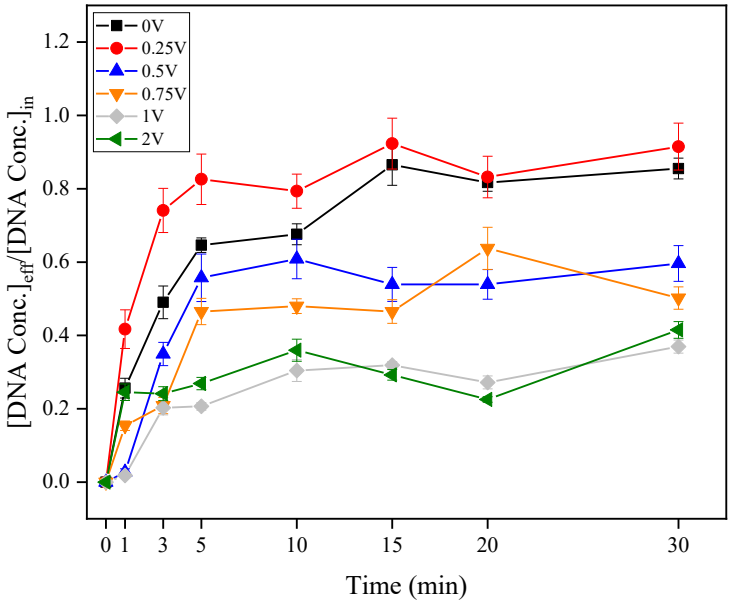

Figure 4-4 Breakthrough curves of bacterial DNA under different potentials. Black squares: breakthrough curve analyzed under 0 V; red circles: breakthrough curve analyzed under 0.25 V; blue triangles: breakthrough curve analyzed under 0.5 V; orange upside down triangles: breakthrough curve analyzed under 0.75 V; grey diamonds: breakthrough curve analyzed under 1 V; green left-pointing triangles: breakthrough curve analyzed under 2 V. Error bars represent the standard error of three replicates.

It took 5 mins for bacterial DNA to reach the breakthrough under 0.75 V and below, and about 1 min and 3 mins to reach the breakthrough under 1 V and 2 V, respectively. Similar breakthrough curves can be observed for filtration of 16s rRNA genes and *erm*80 genes. Real-time qPCR measurements showed that with 2 V to the electrochemical filtration, 0.78-log and 1.84-log removal for 16S rRNA genes and *erm*80 genes were reached after 30 min, respectively, indicating that electrochemical MWCNT filtration was effective to remove ARGs.

Previous SEM images and breakthrough curves illustrated that DNA adsorption occurred during the electrochemical filtration process. To further investigate the electrochemical effect of membrane other than physical adsorption, 16S rRNA genes and *erm*80 genes were quantified by real-time qPCR method to measure the removal of genes.

Significant enhancement in DNA removal was achieved by applying voltages to the MWCNT filtration reactor. Initial experiments were conducted to estimate the removal of bacterial DNA, 16s rRNA genes and *erm*80 genes by the MWCNT membranes in the absence of applied potential. For bacterial DNA, 23% DNA were removed by MWCNT, as shown in Figure 4-5. The removal effeciency of 16S rRNA genes and *erm*80 genes was 30% and 47%, respectively (Figure 4-7 and Figure 4-9). The removal efficiencies increased with increasing electric potentials. The MWCNT filtration achieved 71% removal effciency of bacterial DNA under high voltages, and the removal on 16s rRNA genes and *erm*80 genes were also enhanced by applying voltages to 88% and 98%, respectively. Specifically, DNA solution filtered by 0.75 V resulted in 79% of 16S rRNA genes removed, which was comparable with 87% and 88% under 1 V and 2 V, respectively. On the other hand, filtration of *erm*80 genes under 0.75 V, 1 V, and 2 V led to an average of 91%, 93%, and 98% removal rates. It can be seen from the figures that a considerable increase of the removal efficiency occurred from 0.5 V to 0.75 V, consistent with the oxidation peak around 0.5V in the CV curves. As expected, applying potential had a significant effect on the removal of ARGs.

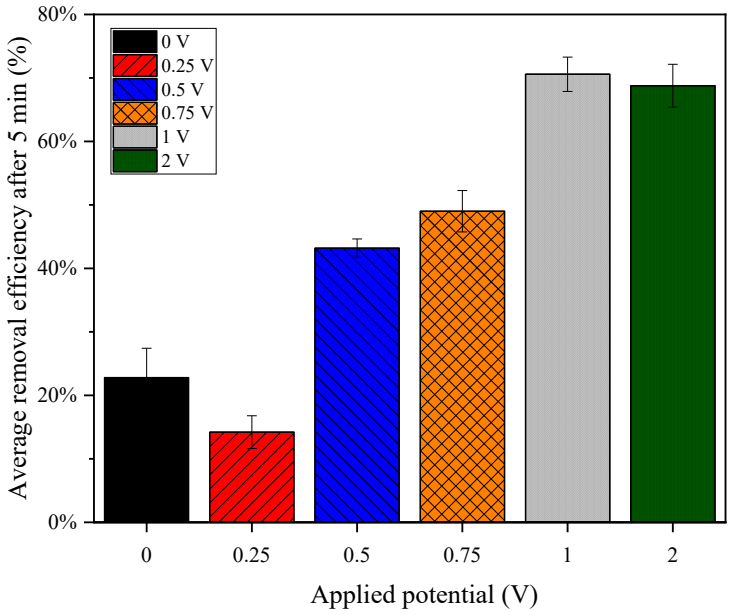

Figure 4-5 Effects of applied potential on removal efficiency of bacterial DNA. Average removal efficiencies of genomic DNA after 5 min under 0 V to 2 V. Error bars represent the standard error of three replicates.

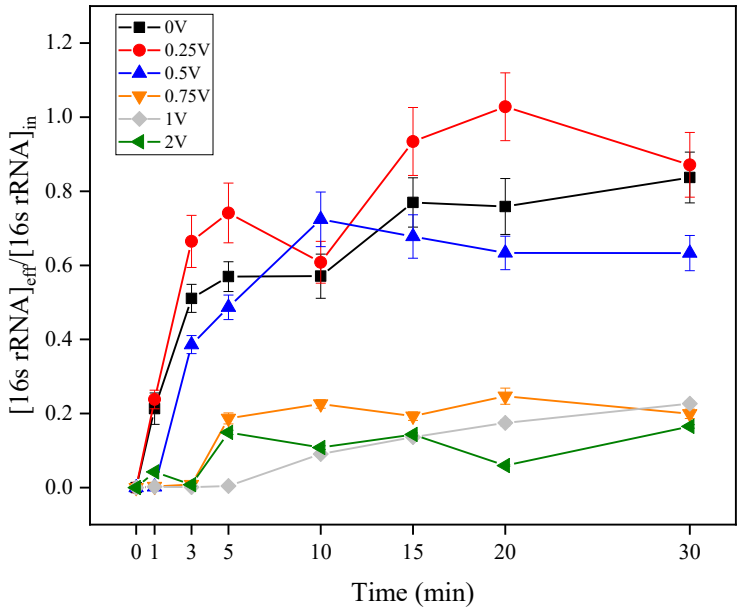

Figure 4-6 Breakthrough curves of bacterial DNA under different potentials. Electrochemical bacterial DNA filtration at potentials of 0 V, 0.25 V, 0.5 V, 0.75 V, 1 V and 2 V. The values indicate the mean of triplicate experiments, and the error lines represent the standard error of triplicate experiments.

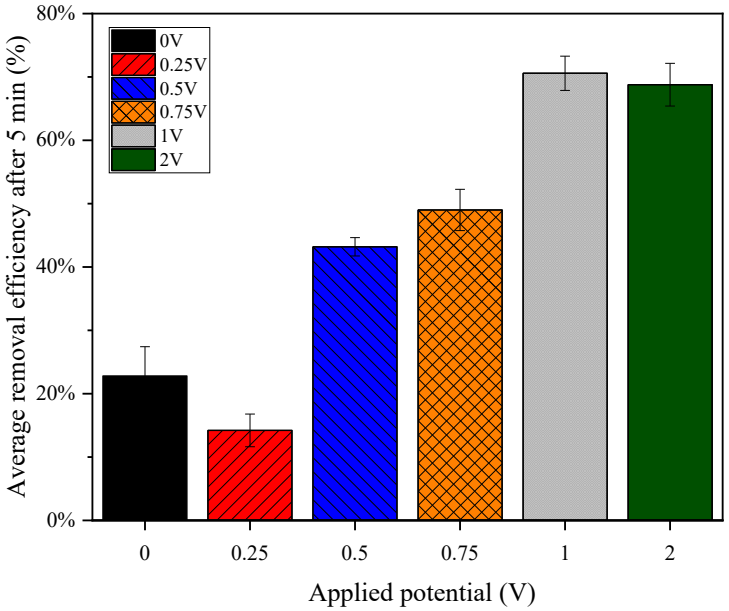

Figure 4-7 Effects of applied potential on removal efficiency of 16S rRNA gene. Average removal efficiencies of 16S rRNA genes after 5 min under 0 V to 2 V. Error bars represent the standard error of three replicates.

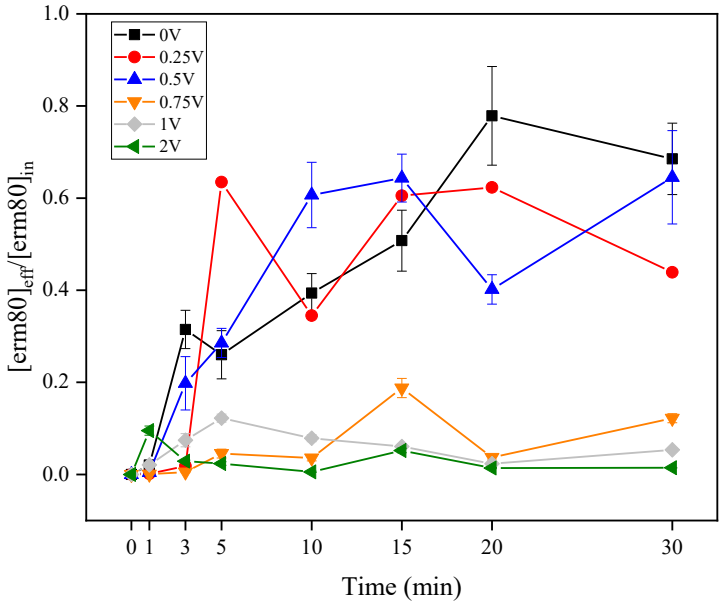

Figure 4-8 Breakthrough curves of *erm*80 gene under different potentials. Error bars represent the standard error of three replicates.

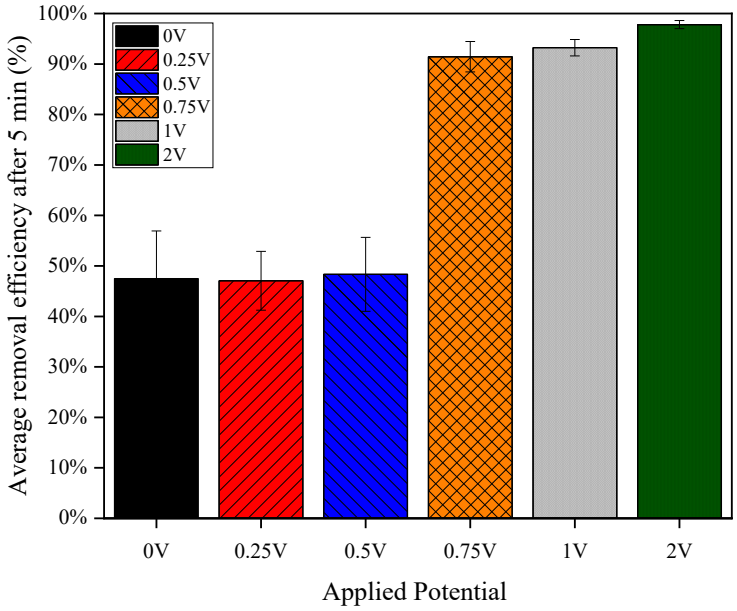

Figure 4-9 Effects of applied potential on removal efficiency of *erm*80 gene. Average removal efficiencies of *erm*80 genes after 5 min under 0 V to 2 V. Error bars represent the standard error of three replicates.

The results showed that high voltage applied to the filtration reactor could boost the attraction of charged DNA to MWCNT membranes, thereby remarkably improving the removal efficiency of DNA. However, when higher voltages (> 1 V) were employed to the reactor, noticeable disruptions on the surface of MWCNT membrane were detected (Figure 4-10), which was likely due to the water electrolysis. Under high electric potentials, the water in the reactor was split into hydrogen gas ($2H^+ + 2e^- \rightarrow H_2$ (gas)) and oxygen gas ($2H_2O$ (liquid) $\rightarrow O_2$ (gas) + $4H^+ + 4e^-$). The cathodic hydrogen evolution reaction and anodic oxygen reaction are two important half-cell reactions for electrochemical water splitting, and they caused the formation of visible air bubbles during electrochemical filtration process which decreased the surface area of MWCNT membrane directly contacting with DNA molecules (Y. Liu et al., 2014). Therefore, the decomposition of water into hydrogen gas and oxygen gas reduced the removal effectiveness of MWCNT filtration reactor. To keep the removal efficiency of DNA and ARGs and prevent fouling, an optimized electric potential of 0.75 V was carried out for further filtration experiments.

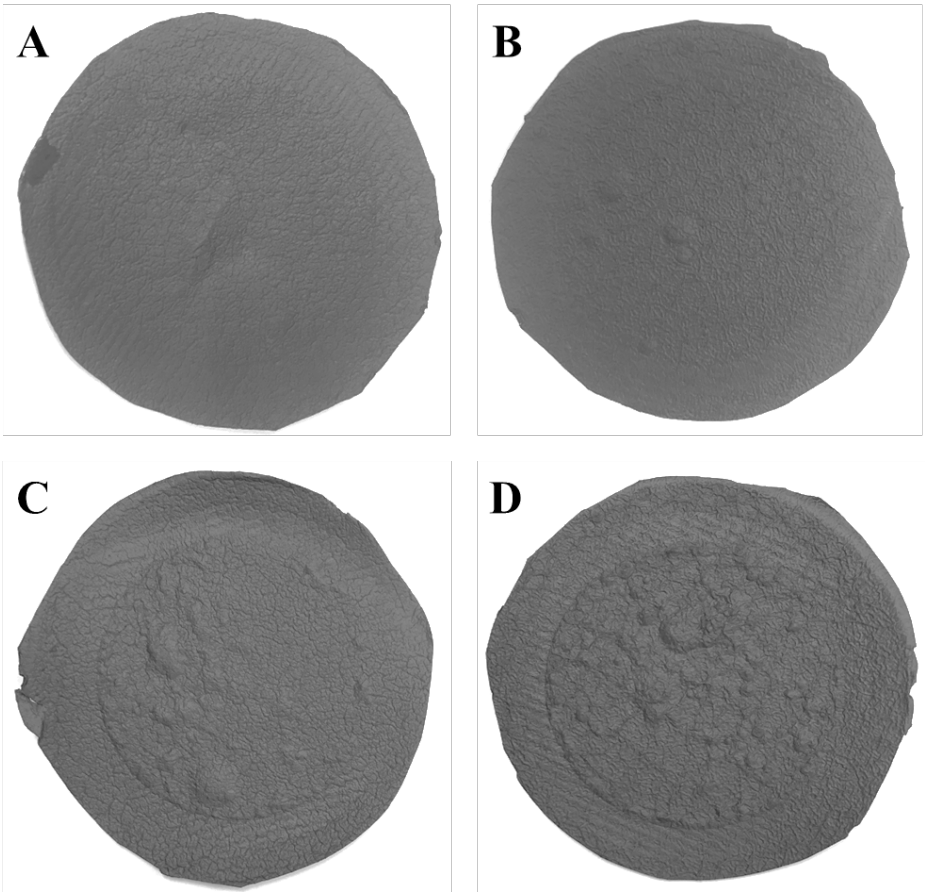

Figure 4-10 MWCNT filters after electrochemical filtration experiments. (A) applied 0 V to the reactor. (B) applied 0.75 V to the reactor. (C) applied 1 V to the reactor. (D) applied 2 V to the reactor.

The mechanisms behind the remarkable increase in the removal of DNA and ARGs in the presence of applied potentials are electrophoresis and subsequent electrolysis of DNA (Rahaman et al., 2012). The MWCNT filtration reactor was externally applied electric potential that improved the transportation of negatively charged DNA molecules to the positively charged anodic MWCNT surface. Meanwhile, the applied potential could assist successful attachment of negatively charged DNA molecules onto the anodic MWCNT filter. Once the DNA molecules attached to the anodic MWCNT filter, the DNA molecules would be oxidized. Similar mechanism of DNA and SWCNT was also observed from a previous study (Zhao & Johnson, 2007), where the positively charged SWCNT attracted the hydrophilic phosphodiester of DNA, enhancing the bonding between SWCNT and DNA. The traveling rate of DNA molecules are depending on their sizes: the smaller the faster. It may explain the higher removal efficiency of ARGs than that of DNA. The *erm*80

genes is located in the plasmid DNA whose size is smaller than that of the genomic DNA. Therefore, plasmid with *erm*80 genes would be attracted to anodic MWCNT filter more easily, hence more oxidized by the filter than larger DNA molecules.

Even though the MWCNT filter was not charged, there was a small fraction of DNA molecules would bind with it due to the DNA structure. Although the hydrophilic phosphate backbones of DNA molecules are not attracted to the uncharged MWCNT filter, the hydrophobic interior of DNA can interact with the hydrophobic surface of uncharged MWCNT filter (Zhao & Johnson, 2007). Thus, the MWCNT filters still had the ability of removing DNA in the absence of applied potentials.

4.3.3.2 Effects of solution pH

The breakthrough curve of DNA filtration under various pH is illustrated in Figure 4-11.

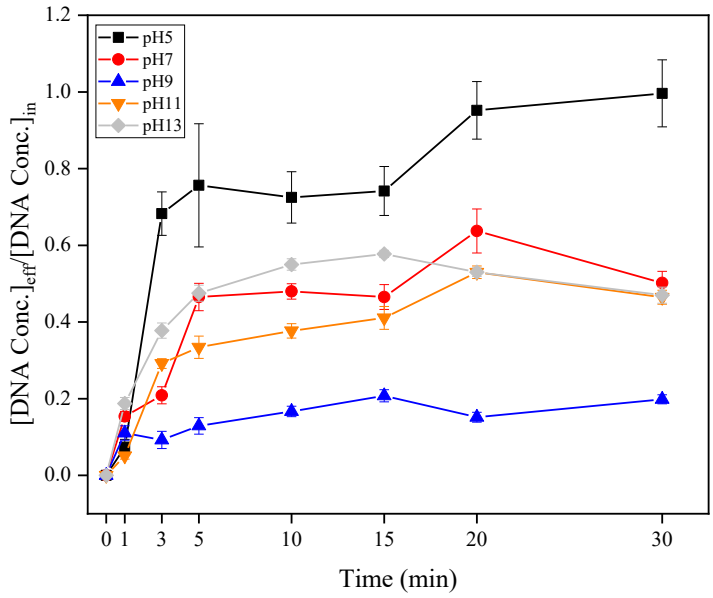

Figure 4-11 Breakthrough curves of bacterial DNA under different pHs. Black squares: breakthrough curve analyzed under pH 5; red circles: breakthrough curve analyzed under pH 7; blue triangles: breakthrough curve analyzed under pH 9; orange upside down triangles: breakthrough curve analyzed under pH 11; grey diamonds: breakthrough curve analyzed under pH 13. Error bars represent the standard error of three replicates.

It only took 1 min for DNA filtration to reach the breakthrough at pH 9, with the highest DNA removal rate among all samples. An increase of naked DNA removal was obtained by applying electric potential at pH 9 and pH 11 (Figure 4-12).

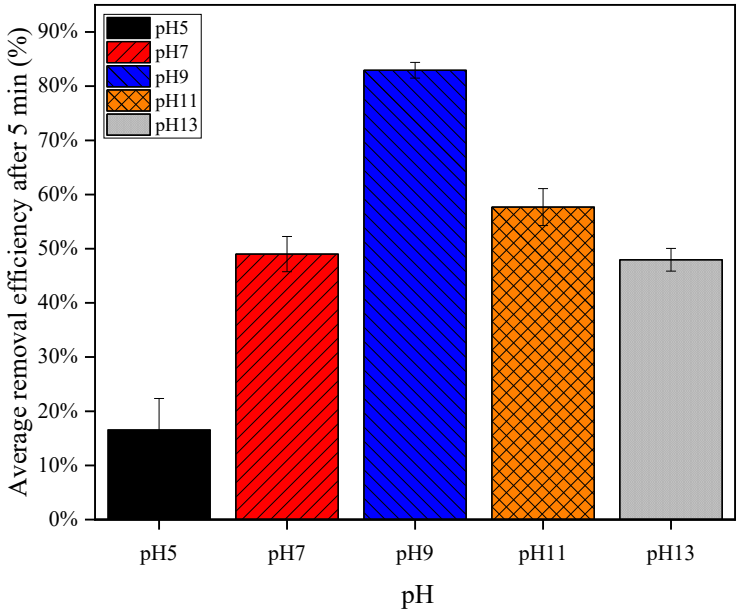

Figure 4-12 Effects of pH on removal efficiency of bacterial DNA. Average removal efficiencies of genomic DNA after 5 min under pH 5 to pH 13. Error bars represent the standard error of three replicates.

With pH 9 solution and 0.75 V, 83% of DNA were removed by the MWCNT filter, compared to 49% in the absence of electric potential, indicating that electrochemical filtration was effective to remove DNA. The absorbance method showed that this electrochemical MWCNT filtration achieved 58% and 48% DNA removed at pH 11 and pH 13, respectively. These results suggest that this water filtration technology could be utilized in environments under various pH conditions.

Within the tested pH of 5 to 13 in this study, the 16S rRNA removal was not significantly changed except at pH 5 (Figure 4-13 and Figure 4-14). The removal of 16s rRNA genes was achieved the lowest (0.63-log removal) after 5-min filtration at pH 5. The removal efficiency of 16S rRNA genes increased when pH was between 7 and 9, where the removal efficiency was 79% and 83%, respectively. Log 16s rRNA genes removal at 5-min filtration reduced from 1.08 to 0.85 as the solution pH increased from 9 to 13.

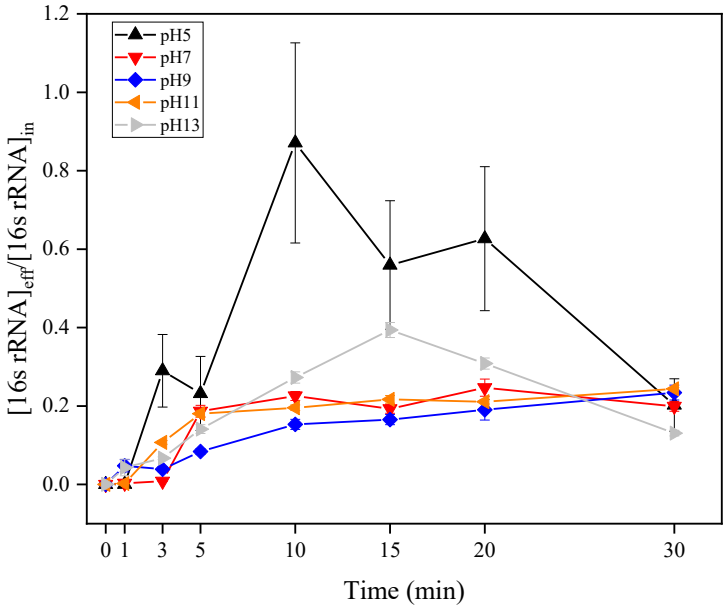

Figure 4-13 Breakthrough curves of 16S rRNA gene under different pHs. Error bars represent the standard error of three replicates.

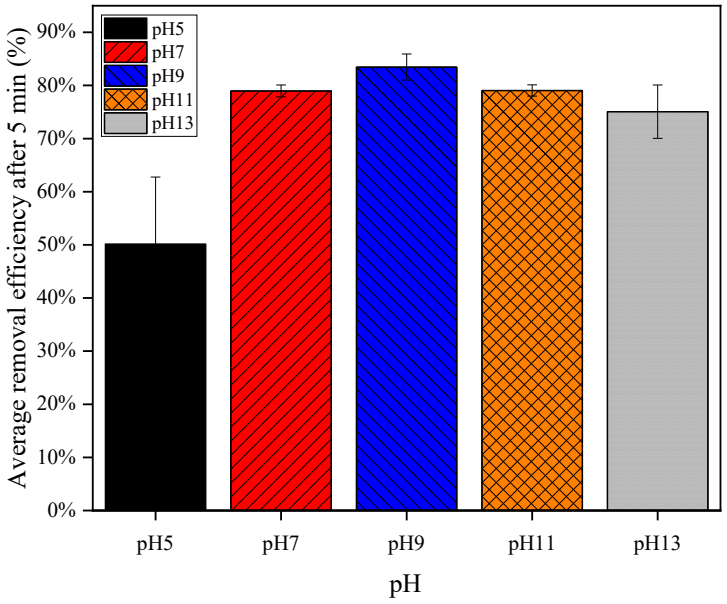

Figure 4-14 Effects of pH on removal of 16S rRNA gene. Error bars represent the standard error of three replicates.

The effects of pH on removal efficiency of ARG was quite small, except at pH 5. As shown in Figure 4-15, *erm*80 resistant genes removal efficiency decreased with increasing pH over the range from pH 7 to pH 13.

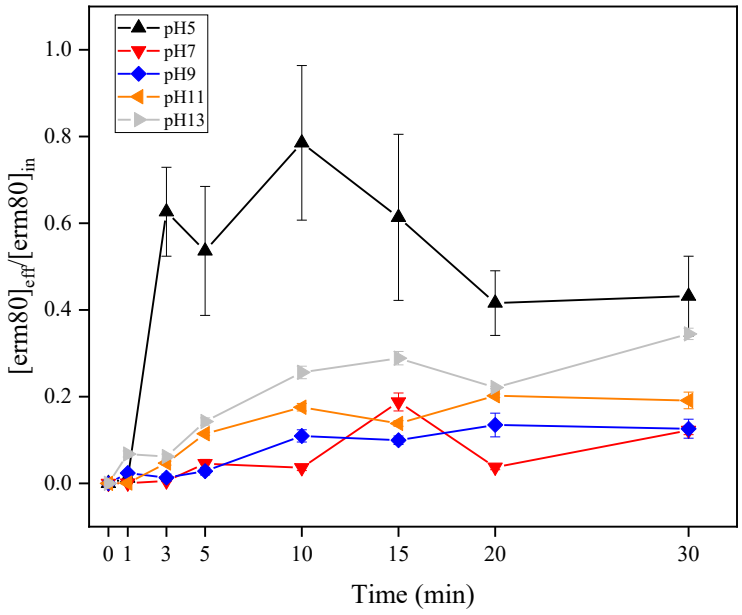

Figure 4-15 Breakthrough curves of *erm*80 under different pHs. Error bars represent the standard error of three replicates.

It is clear from Figure 4-16 that the removal efficiency of *erm*80 genes at pH 5 was 47% lower than that of *erm*80 genes at pH 7. The average removal efficiency of *erm*80 genes by electrochemical MWCNT filtration technology from pH 7 to pH 13 was 85%, indicating that this ARGs removal treatment can be effectively applied in the real environments. The possible mechanisms of adding acid and alkali to this electrochemical filtration system may be explained by the reactions among H^+ ions, OH^- ions, and DNA. The MWCNT filter was charged with positive charges, thus attracting negative-charged DNA molecules, resulting in a high removal rate of DNA. Once the additional acid is added, the DNA solution is occupied with H^+ ions, which neutralize the electrical charge of DNA if bonded, hence lower the removal rate. On the other hand, with pH increasing, more OH^- ions are available in the solution. Two mechanisms of contradictory effects occur at the same time. One is that some of the OH^- ions are bonded with DNA through O-H covalence bond, exaggerating the negative charge of DNA and enhancing the removal rate. Hydroxide ions also competes with DNA as both carry negative charge while attracted by the

MWCNT. Therefore, with a moderate amount of hydroxide ions present, the removal rate increases due to higher charges of DNA, as seen in the case of pH 9. As the pH increases, the competing effect dominates and decreases the DNA removal rate.

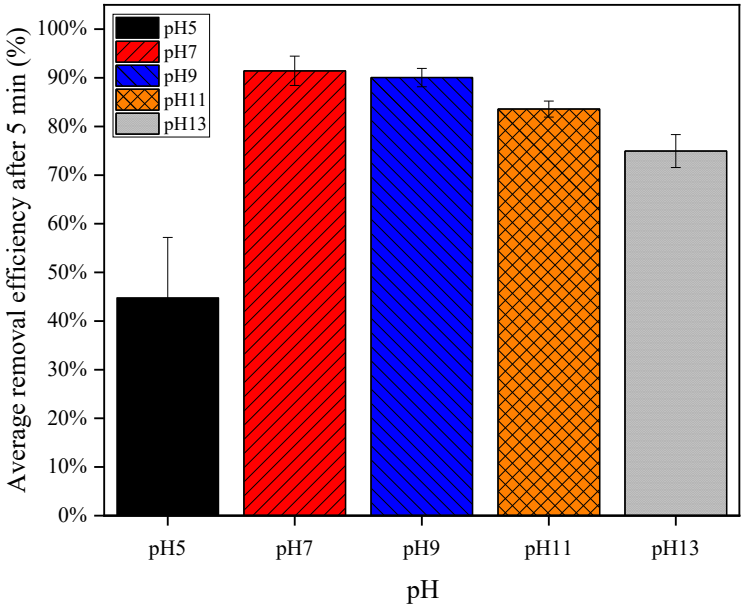

Figure 4-16 Effects of pH on removal efficiency of *erm*80 gene. Error bars represent the standard error of three replicates.

4.3.3.3 Effects of phosphate

A comparison of DNA removal by MWCNT filtration in the absence and presence of phosphate ions is displayed in Figure 4-17. The addition of phosphate ions resulted in a significant reduction on DNA removal. For example, adding phosphate ions made a sharp increase of $[\text{DNA concertation}]_{eff}/[\text{DNA concertation}]_{in}$ with filtration time until a plateau of 24% removal was reached after 5 min. Specifically, filtering DNA solution with 0.125 M Na_2HPO_4 solution, the DNA removal (21% efficiency) was significantly lower than the DNA removal (49% efficiency) after filtering with 0 M Na_2HPO_4 solution (Figure 4-18). No obvious differences of DNA removal can be found from 0.0313 M to 0.125 M Na_2HPO_4 solution.

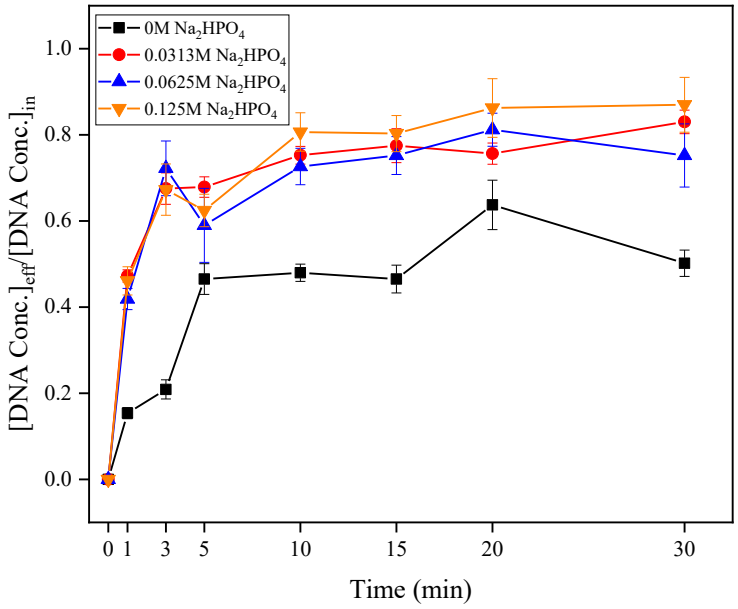

Figure 4-17 Breakthrough curves of bacterial DNA under exposure of phosphate. Black squares: breakthrough curve analyzed with 0 M Na_2HPO_4 solution; red circles: breakthrough curve analyzed with 0.0313 M Na_2HPO_4 solution; blue triangles: breakthrough curve analyzed with 0.0625 M Na_2HPO_4 solution; orange upside down triangles: breakthrough curve analyzed with 0.125 M Na_2HPO_4 solution. Error bars represent the standard error of three replicates.

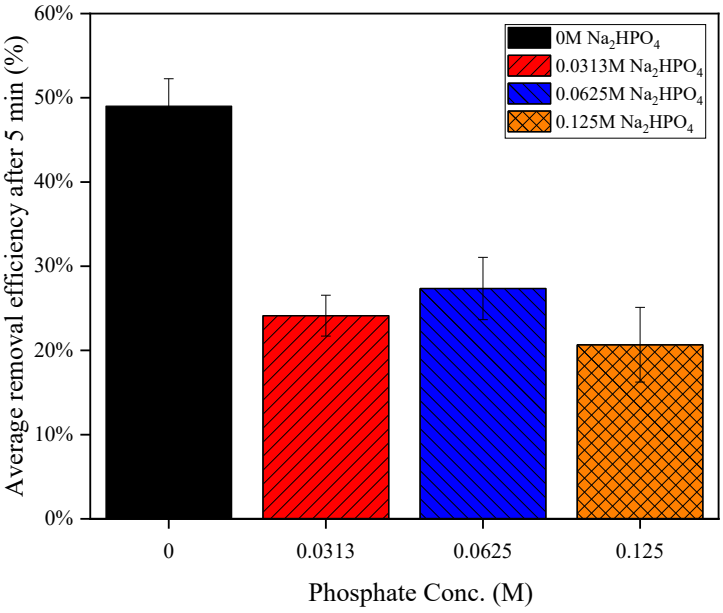

Figure 4-18 Effects of phosphate on removal efficiency of bacterial DNA. Average removal efficiencies of genomic DNA after 5 min with 0 M to 0.125 M Na_2HPO_4 solution Error bars represent the standard error of three replicates.

The removal efficiency of DNA increased from 79% to 95% over the tested phosphate concentrations of 0 M to 0.0625 M (Figure 4-19). Addition of higher level of phosphate ions (0.125 M) significantly reduced the DNA removal to 44% (Figure 4-20).

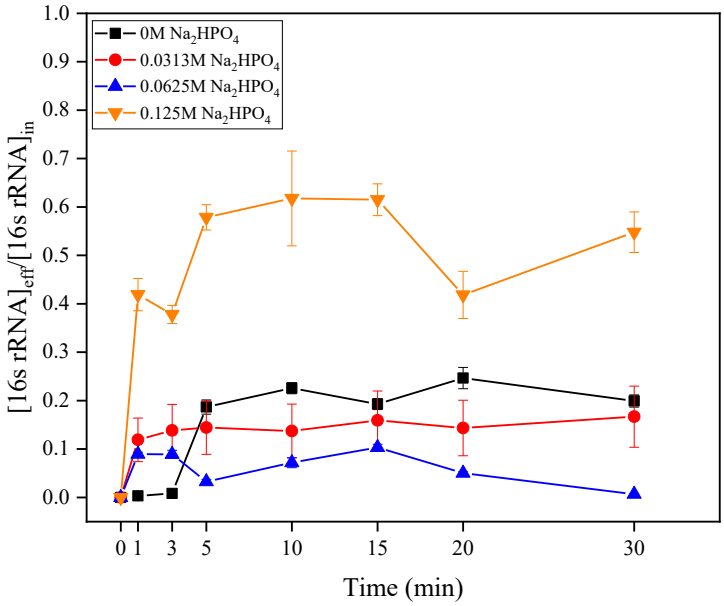

Figure 4-19 Breakthrough curves of 16S rRNA gene under exposure of phosphate. The influent DNA-Na_2HPO_4 concentrations were 0 M, 0.0313 M, 0.0625 M, and 0.125 M. Error bars represent the standard error of three replicates.

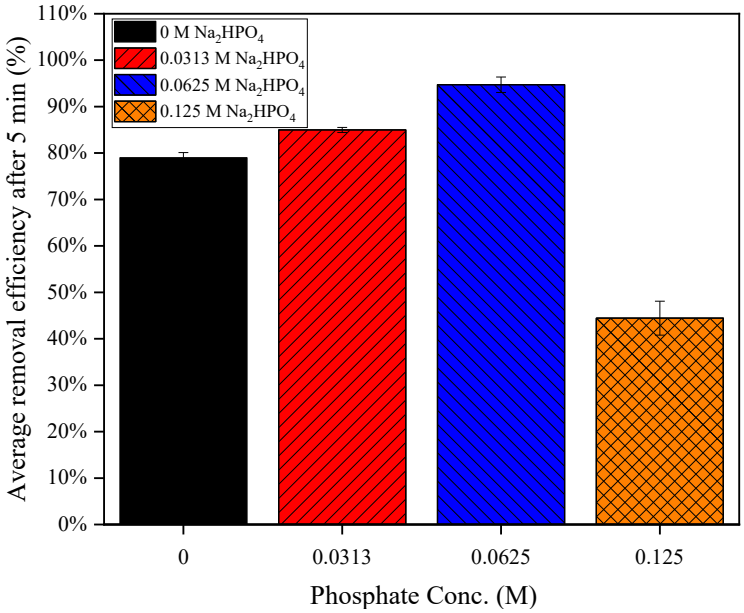

Figure 4-20 Effect of phosphate on removal efficiency of 16S rRNA gene. Error bars represent the standard error of three replicates.

Further increases in phosphate concentration, positively affected the removal efficiency on the MWCNT filters. Similar results were observed that the removal efficiencies of DNA and *erm*80 genes with 0.0625 M Na_2HPO_4 solution were the highest (95% and 37%, respectively) in the presence of phosphate ions but still lower than the removal efficiencies (79% removal for 16S rRNA genes and 91% removal for *erm*80 genes) without phosphate ions (Figure 4-20 and Figure 4-22).

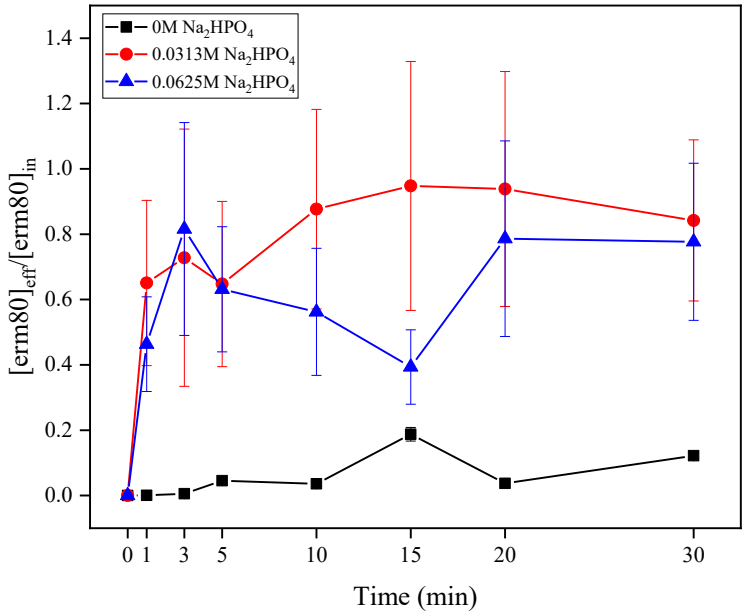

Figure 4-21 Breakthrough curves of *erm*80 gene under exposure of phosphate. Error bars represent the standard error of three replicates.

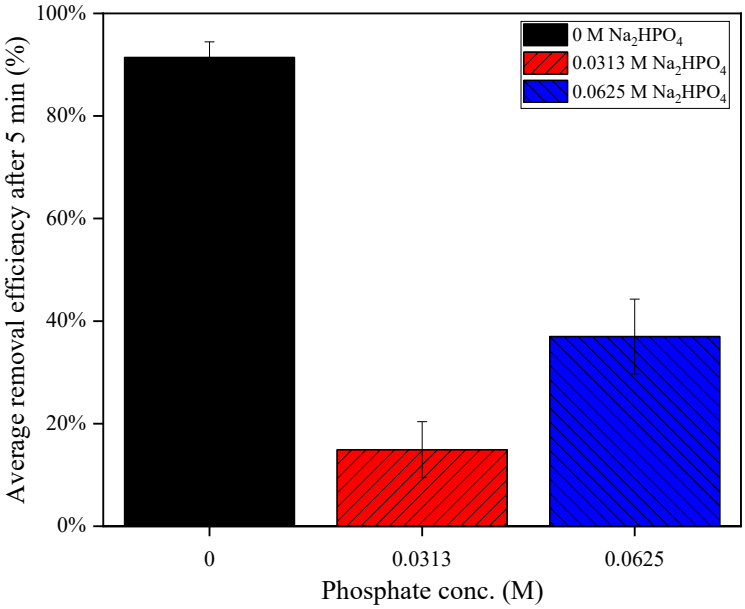

Figure 4-22 Effects of phosphate on removal efficiency of *erm*80 gene. Error bars represent the standard error of three replicates.

No gene copy numbers of *erm*80 genes were obtained with 0.125 M Na_2HPO_4 by qPCR due to the high salt concentration of the qPCR solution. Because of the high precision and sensitivity of real-

time PCR method, a variety of factors like salt concentrations, pH, phenol and alcohol from DNA purification method, DNA concentrations, or other unknown cell constituents could impact the fluorescence emission of qPCR reaction (Bustin, 2004). To eliminate the inhibition of phenol and alcohol in our genomic DNA samples, A_{260}/A_{280} ratios of DNA samples were measured by NanoDrop UV-Vis spectrophotometer. The ratios were from 1.8 to 2.0, indicating that the DNA samples were pure enough for qPCR experiments. The reasons for the low fluorescence intensity in this experiment could be the high concentration of both Na_2SO_4 (as the background electrolyte) and Na_2HPO_4 in the reaction, and the low concentrations of DNA. Further optimizations of the inhibitor concentrations should be conducted in the future.

The reason for the changes of DNA removal is not clear but we hypothesize two possible mechanisms could affect the DNA and ARGs removal in the electrochemical MWCNT filtration. First, increasing concentration of Na^+ could make the electrostatic repulsion among subunits of DNA increased, thus the subunits of DNA could move closer to make more compacted DNA molecules (Nguyen & Elimelech, 2007). Therefore, the electrostatic forces between compacted DNA molecules and anodic MWCNT filter become less significant and the removal efficiency of DNA removal will also be reduced. Second, considering the three pK_a values of phosphoric acid, which are 2.16, 7.21, and 12.32 (CRC, 2004), and the pH of the DNA solution in this study as 7.25 to 7.33, $H_2PO_4^-$ and HPO_4^{2-} were dominant in the solution. The presence of phosphate ions may compete with the phosphate groups of DNA's backbones during the electrochemical filtration process, so that impact the DNA removal. The mechanisms of this reaction are complicated, and more tests should be done in the future.

4.3.3.4 Effects of NOM

Humic acid was chosen to simulate the organic matter in natural environments because of the prevalence of humic-like substances in aquatic systems (Barker et al., 1999). As shown in Figure 4-23, DNA filtration performances were similar under 5ppm, 10ppm, and 20ppm of NOM, all with lower DNA removal efficiency than that without the interference of NOM.

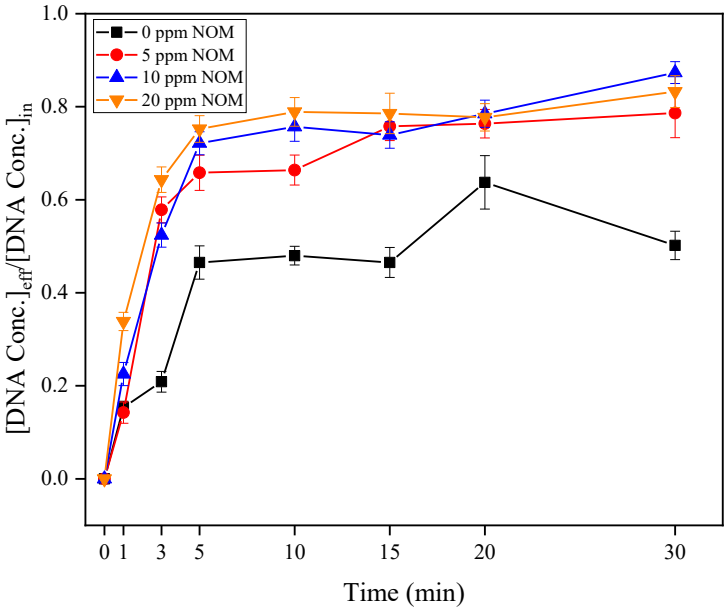

Figure 4-23 Breakthrough curves of bacterial DNA under exposure of NOM. Black squares: breakthrough curve analyzed with 0 ppm NOM solution; red circles: breakthrough curve analyzed with 5 ppm NOM solution; blue triangles: breakthrough curve analyzed with 10 ppm NOM solution; orange upside down triangles: breakthrough curve analyzed with 20 ppm NOM solution. Error bars represent the standard error of three replicates.

The results in Figure 4-24 display that bacterial DNA removal decreases with higher concentrations of NOM, in spite of a marginal effect after 5ppm. The highest removal efficiency of DNA was 49% in the absence of NOM, and only 21% bacterial DNA removal was found when the NOM concertation reached the relatively high level of 20 ppm.

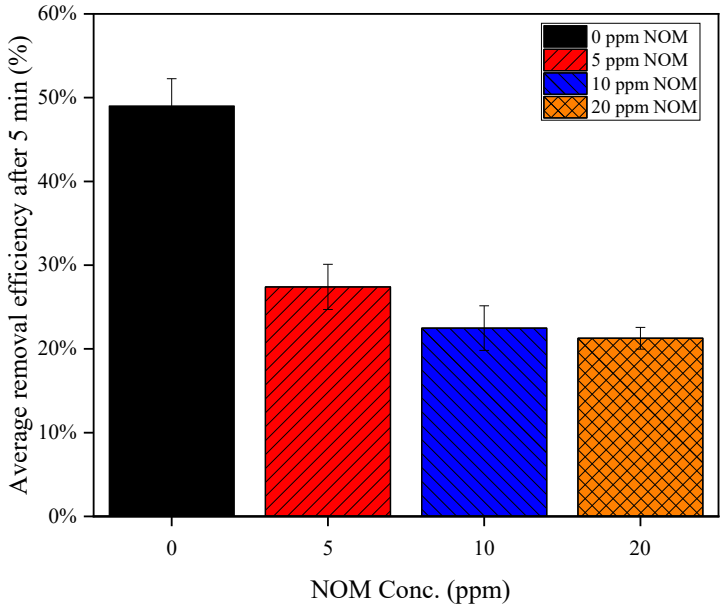

Figure 4-24 Effects of NOM on removal efficiency of bacterial DNA. Average removal efficiencies of genomic DNA after 5 min with 0 ppm to 20 ppm NOM solution. Error bars represent the standard error of three replicates.

The relatively lower removal of DNA and ARGs in the presence of NOM may be explained by the shielding effect of the NOM-DNA complex, protecting the complex from oxidation by MWCNT filters. Genomic DNA were more prone to bond with organic matters, though there were anodic MWCNT membrane in the system. Both chromosomal and plasmid DNA are rapidly adsorbed and bound with humic acid (Crecchio et al., 1998). The DNA-NOM complexes were persistent in the environment, thus more resistant to DNA degradation than free DNA. This may explain the lower removal efficiency of DNA under higher NOM concentrations that extracellular DNA in the filtration system would more likely to bond with humic acid first due to divalent cation complexation with the carboxylate groups of humic acid and the DNA's phosphate backbones (Crecchio et al., 2005). Subsequently, the complexes of DNA and NOM were difficult to attract to the anodic MWCNT membrane, resulting in lower DNA removal efficiencies.

The removal efficiency of DNA with NOM was evaluated by quantifying the gene copy numbers of 16s rRNA genes from the MWCNT filter (Figure 4-25). A significant 16S rRNA gene copy numbers, 1.90×10^7 genes/mL, 8.76×10^6 genes/mL, and 2.96×10^7 genes/mL, were removed by the MWCNT membrane with the addition of 5 ppm, 10 ppm, and 20 ppm NOM, respectively.

Additional analysis revealed that as NOM concentration rose, the impact of NOM on the removal of 16S rRNA and *erm*80 genes became more vital (Figure 4-26, Figure 4-27, Figure 4-28). Specifically, 20 ppm NOM led to 99% removal efficiency of 16s rRNA. Furthermore, the absence of NOM in the filtration reactor was found to have the most significant impact on the removal of *erm*80 genes which was 91% whereas 68% removal efficiency was observed when 20ppm NOM solution was applied.

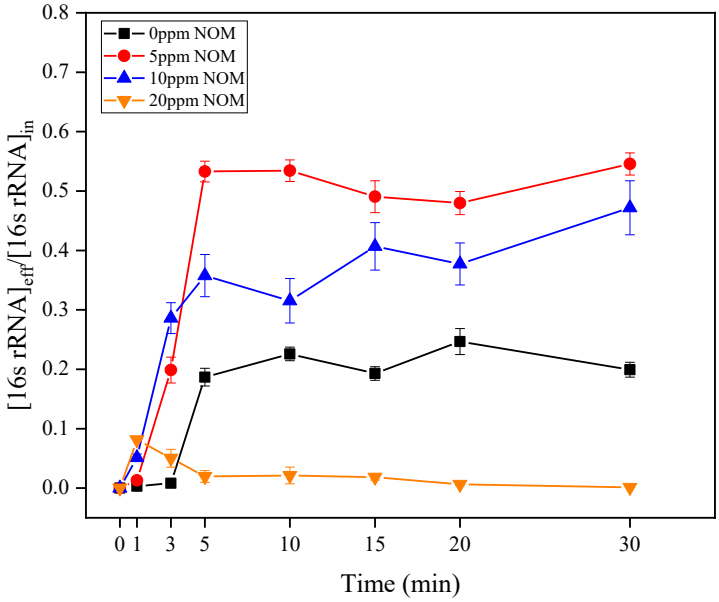

Figure 4-25 Breakthrough curves of 16S rRNA gene under exposure of NOM**.** The influent DNA-NOM concentrations were 0 ppm, 5 ppm, 10 ppm, 20 ppm. Error bars represent the standard error of three replicates.

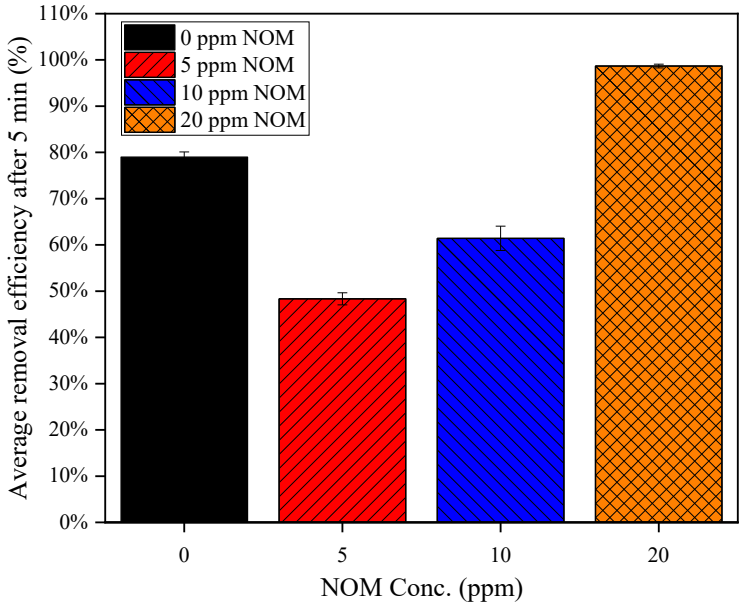

Figure 4-26 Effect of NOM on removal efficiency of 16s rRNA gene. Error bars represent the standard error of three replicates.

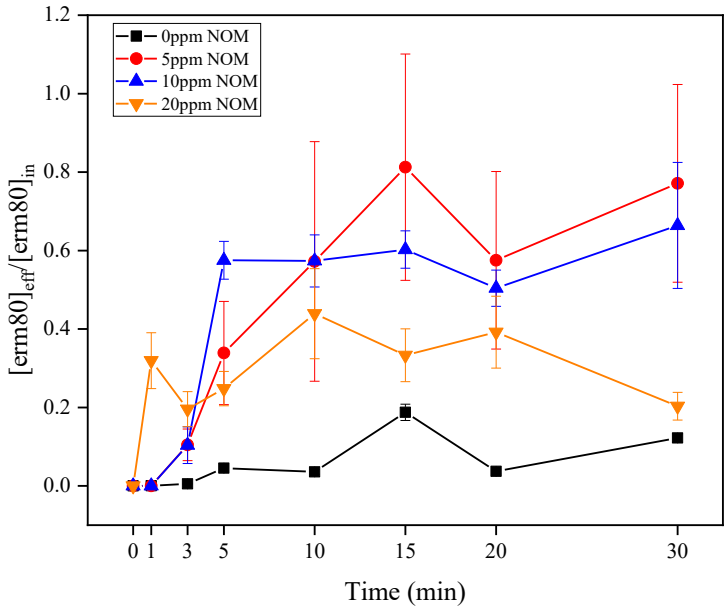

Figure 4-27 Breakthrough curves of *erm*80 gene under exposure of NOM. Error bars represent the standard error of three replicates.

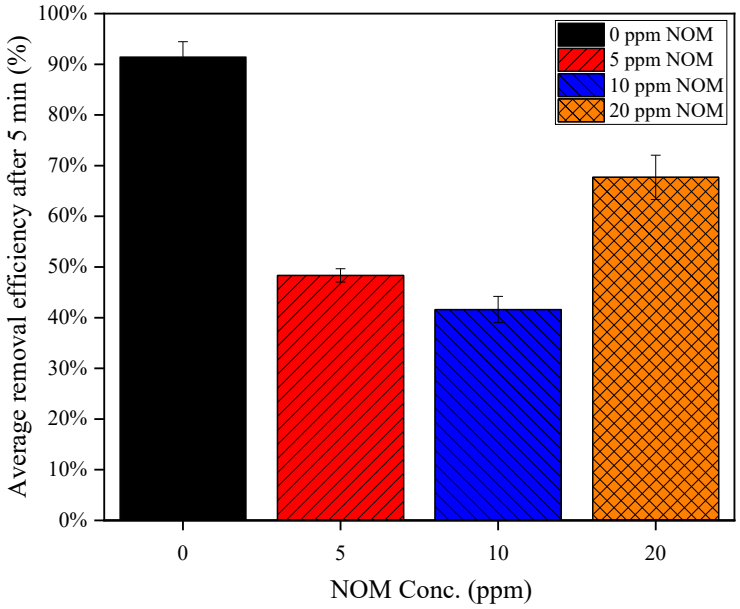

Figure 4-28 Effects of NOM on removal efficiency of *erm*80 gene. Error bars represent the standard error of three replicates.

4.4 Conclusion

Overall, the results of this study showed that electrochemical MWCNT filtration is a promising technology for the removal of DNA and ARGs in environments.

Contrary the ideas that DNA molecules are small and flexible which may go through a membrane pore (Marko et al., 2011), we demonstrated that both extracellular DNA and ARGs can be effectively removed simultaneously by electrochemical MWCNT filtration under electric potentials as low as 0.75V. The best removal efficiency reached 88%, and 98% for DNA and ARGs, respectively, while remaining a similarly high level under a wide range of conditions such as pH, phosphate, and NOM.

Furthermore, additional phosphate and NOM that exist in the environments could further enhance the removal efficiency of 16s rRNA genes to 95% and 99%, respectively. With a robust setup and low-cost operation, this water treatment method proves to be efficient for removing DNA and ARGs in both mild natural environments and harsh contaminated water systems.

CHAPTER 5. CONCLUSIONS AND FUTURE WORK

5.1 Conclusions

Our study is the first one to look into the effects of sub-MIC level of antibiotics on the horizontal transfer of ARGs between environmental bacteria at environmentally relevant concentrations. Astonishingly, a low erythromycin concentration of 0.4 ng/L significantly enhanced conjugative transfer frequency of *erm*80 genes, which may be attributed to the induction of the SOS response. Additionally, fitness cost and biofilm formation by the sub-MIC of erythromycin also increased ARG transfer frequency. The study revealed the long-neglected health risks of the extremely low concentration of antibiotics in the natural environments. It is urgent and crucial for the scientific community to realize, stop and alleviate the health risk that was not well characterized.

Multiple approaches were adopted to detect the occurrence and development of ARGs. The FISH technique showed advantages of identifying *erm* gene expression in the recipients, regardless of VBNC state, which both traditional culture-based approach and qPCR failed to do. The FISH method is capable of assessing the potential risk of the HGT process, in both lethal level and extremely low concentration of antibiotic resistance. The underestimation of the HGT transfer with culture-based approach observed in this study is rather alarming. Since culture-based approach is often regarded as the standard method for bacteria cell counting, a correction regarding to the VBNC state is important for an accurate environmental health risk assessment.

The effect of MWCNT on HGT was studied. Interestingly, the addition of CNT could lead to different effect on the development of antibiotic resistance. It was found that CNT encouraged HGT above 1 MIC while the contrary effect was observed below 1 × MIC.

To solve the HGT problems in natural environments, the MWCNT electrochemical filtration method was studied and evaluated. Both extracellular DNA and ARGs were effectively removed. The setup can operate under electric potentials as low as 0.75V. The best removal efficiency reached 88%, and 98% for DNA and ARGs, respectively, while remaining a similarly high level under a wide range of conditions such as pH, phosphate, and NOM. Furthermore, additional

phosphate and NOM that exist in the environments could further enhance the removal efficiency of 16S rRNA genes to 95% and 99%, respectively. With a robust setup and low-cost operation, this water treatment method proves to be efficient for removing DNA and ARGs in both mild natural environments and harsh contaminated water systems.

5.2 Future work

Further quantitative studies of the VBNC state are beneficial under various concentrations of antibiotics. As demonstrated by FISH and culture-based approach, a large portion of the cells may enter into the VBNC state under the selective pressure of either growth competition or antibiotics. For some bacteria that were thought as sensitive to antibiotics over MIC, it is possible that the observation of the lack of colonies using culture-based approach is a result of VBNC states rather than losing vitality permanently. An underestimated MIC due to this reason will expose sensitive bacteria to sub-MIC, aggregating the forming of antibiotic resistance. Therefore, it is crucial to accurately measure and identify the true "MIC" compared to the traditional culture-based method.

The opposing results that CNTs create regarding to the HGT can be further studied. We believe that CNT may pose additional selective pressure for the spread of ARGs. Their effects of horizontal gene transfer should be further investigated.

Additional studies and optimizations can be performed for the removal of DNA and ARGs using MWCNT filtration. First, the mechanism of DNA molecules with the MWCNT membrane, and the interactions among DNA, NOM, and MWCNT membrane during electrochemical filtration will be further determined. Membrane materials and other environmental impact factors on DNA and AGRs removal will be optimized. The durability of MWCNT filter under high applied potentials will be quantified and improved. The removal of other small biomolecules by this electrochemical MWCNT filtration technology will be conducted. Furthermore, it is plausible to add surface treatment to the MWCNT filter to increase its performance and reliability.

Nutrients have major influences on the microbial community and the levels of antibiotic resistance bacteria are correlated with the nutrients. In aquatic environments, the survival of fecal indicator bacteria could be monitored for a period of 90 days in the sediments providing favorable nutrient

conditions, and the nutrient concentrations could also help to disseminate ARGs via HGT (Haller et al., 2009). A previous study indicated that the concentrations of nitrogen and phosphorus were strongly correlated with the emergence of antibiotic resistance phenotypes in aquatic environments (Ali et al., 2016). Recently, USEPA provided numeric nutrient criteria for total nitrogen and total phosphorus in three main water bodies (river/streams, lakes/reservoirs, and estuaries) to protect waters from impairment (Agency, 2017). The potential risk on spread of ARGs from exposure to low levels of nutrient pollutants has not been examined. Therefore, efforts are needed to understand the sub-inhibitory concentrations of nutrient that contribute to the spread and propagation of ARGs which may help to identify new and effective strategies to regulate pollution.

It is still unknown whether plasmid with ARGs could transfer between bacteria under nutrient starvation, and the resistant gene expression of VBNC cells has not been well studied. Further study on nutrient starvation may provide information on resistant gene expression for VBNC cells, especially MLS_B resistant genes.

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