INFLUENCE OF TRACE ERYTHROMYCIN AND ERYTHROMYCIN-H_2O ON MICROBIAL CONSORTIA IN SEQUENCING BATCH REACTORS (SBRS)

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Summary

In the 1940s, antibiotics were firstly applied as clinical medicine in treating infections. Initially, the efficiency of antibiotics in killing pathogenic bacteria has led many to believe that antibiotics would be potent to eliminate all infectious diseases from human beings. Disappointedly, the successful use of the therapeutic antibiotics has been compromised by the emergence and rapid dissemination of resistant pathogens, especially multi-drug resistant microorganisms. The recent development of antibiotic resistance in pathogens is believed to be a result of anthropogenic activities, the massive production and application of antibiotics in the disease treatment and growth promotion. However, the lack of knowledge on the evolution of antibiotic resistance genes and environmental roles of antibiotics has hampered efforts to prevent and control the proliferation of antibiotic resistance. This drives the need to investigate antibiotic influence on wastewater treatment plants (WWTPs), which are the main collection pools of anthropogenic discharges of antibiotics and antibiotic resistance genes. The influences of antibiotics on micro-ecosystem of WWTPs include ecological function disturbance, resistance selection and phylogenetic structure alteration, which are the focuses of this study.

This dissertation firstly demonstrated the effects of antibiotic erythromycin (ERY, 100 μg/L) and its derivative ERY-H_2O (50 μg/L) on the disturbance of ecological functions, including carbon, nitrogen (N), and phosphorus (P) removal in sequencing batch reactors (SBRs) (chapter 3). The findings in this study show that the effects of ERY or ERY-H_2O on the removal of carbon, N, and P were negligible when compared with the control reactor. However, ERY and ERY-H_2O had pronounced effects on the community composition of bacteria associated with N and P removal, leading to a decrease in diversity and a change in abundance. Therefore,

the presence of ERY or ERY-H_2O (at μg/L levels) shifted the microbial community and selected antibiotic resistant bacteria, which may account for the negligible influence of the antibiotic ERY or its derivative ERY-H_2O on carbon, N, and P removal in the SBRs.

This thesis further identified the causal correlation of trace ERY (100 μg/L) or ERY-H_2O (50 μg/L) with antibiotic resistance proliferation (chapter 4). Erythromycin resistance genes were screened on microbial consortia of SBRs after one year acclimation to ERY (100 μg/L) and ERY-H_2O (50 μg/L). Results revealed that the effects of ERY and ERY-H_2O on the proliferation of antibiotic resistance genes were limited to esterase gene *ere*A. The above consortia of SBRs were also applied to evaluate their capability to esterify ERY through *ere*A gene. Results showed that ERY was bio-transformed into six products by microbes acclimated to ERY (100 μg/L). However, ERY could not be bio-transformed by those microbes acclimated to ERY-H_2O (50 μg/L), which may be due to the less amounts of proliferated *ere*A gene. Biodegradation of ERY required the exogenous carbon source (e.g., glucose) and nutrients (e.g., nitrogen, phosphorous) for assimilation. However, overdosed ammonium–N (>40 mg/L) inhibited degradation of ERY. *Zoogloea,* a type of biofilm-forming bacteria, became predominant in the process of ERY esterification, suggesting that the input of ERY can induce biofilm resistance to antibiotics. This study highlighted that lower μg/L level of ERY or ERY-H_2O in the environment is able to encourage the expansion of resistance genes in microbes.

In chapter 5, the microbial consortia in the SBRs fed with ERY (100 μg/l) or ERY-H_2O (50 μg/l) were analyzed in terms of phylogenetic structure alteration based on 16S rRNA genes, including terminal restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE), and microarrays

(PhyloChip). Results revealed that both ERY and ERY-H_2O markedly altered the composition and structure of the microbial communities in similar inhibitory and selective spectrum when comparing with the control SBR. The Gram-positive Actinobacteria and Gram-negative Proteobacteria were inhibited in terms of both diversity and abundance. The abundance-enriched bacteria belonged to the TM7 phylum and the β-Proteobacteria subphylum (within the genera of *Azonexus, Dechloromonas, Thauera,* and *zoogloea* under the Rhodocyclaceae family, and the *Nitrosomonas* genus). The enriched *zoogloea* are capable of forming biofilm to resist antibiotics, and other enriched Rhodocyclaceae (*Azonexus*, *Thauera,* and *Dechloromonas*) and the *Nitrosomonas* are able to reduce nitrate and oxidize ammonium in order to eliminate these toxic nitrogenous substances accumulated in the biofilm. This is known as biofilm resistance to antibiotics. With phylogenetic analysis on uncultured samples, the results of this study suggested that low levels of ERY or ERY-H_2O can alter microbial communities via the inhibition of sensitive bacteria and the enrichment of biofilm antibiotic resistant bacteria.

In summary, low dose of antibiotics and their derivatives play significant roles in selecting resistant bacteria and proliferating resistance genes among microbes. With the increasing usage of recycled wastewater (e.g., as potable and non-potable water sources), sub-inhibitory concentrations of antibiotics in WWTPs might pose potential risks to human health.

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Abbreviations

AOMs	Ammonium Oxidizing Microorganisms
bp	Base Pair
COD	Chemical Oxygen Demand
Cy5	Cyanine 5
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic Acid
DO	Dissolved Oxygen
ERY	Erythromycin
ERY-H_2O	Dehydrated Erythromycin
EPS	Extracellular Polymeric Substances
GAOs	Glycogen Accumulating Organisms
HRT	Hydraulic Retention Time
LC-MS	Liquid Chromatography Mass Spectrometry
LC-MS-MS	Liquid Chromatography Tandem Mass Spectrometry
MM	Mismatch
MR	Mother Reactor
N	Nitrogen
NMDS	Nonmetric Multi-Dimensional Scaling

NOB	Nitrite Oxidizing Bacteria
nt	Nucleotide
OTU	Operational Taxonomic Unit
P	Phosphorous
PAO	Poly-P Accumulating Organisms
PCR	Polymerase Chain Reaction
PM	Perfect Match
QS	Quorum-Sensing
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
SBR	Sequencing Batch Reactor
SRT	Solid Retention Time
STPs	Sewage Treatment Plants
ThOD	Theoretical Oxygen Demand
TOC	Total Organic Carbon
T-RFLP	Terminal Restriction Fragment Length Polymorphism
T-RFs	Terminal Restriction Fragments
WWTPs	Wastewater Treatment Plants

Publications

Journal articles

1. **Fan, C.**, Lee, P.K.H., Ng, W.J., Alvarez-Cohen, L., Brodie, E.L., Andersen, G.L., He, J., 2009. Influence of trace erythromycin and erythromycin-H_2O on carbon and nutrients removal and on resistance selection in sequencing batch reactors (SBRs). Applied Microbiology and Biotechnology 85(1), 185-195.
2. **Fan, C.,** He, J., 2011. Proliferation of antibiotic resistance genes in microbial consortia of sequencing batch reactors (SBRs) upon exposure to trace erythromycin or erythromycin-H_2O. Water Research 45(10), 3098-3106.

Conference presentations

1. **Fan, C.**, He, J., 2008. Influences of erythromycin and erythromycin-H_2O on aerobic sequencing batch reactor (SBR). American Society for Microbiology's 108^{th} General Meeting. Boston, Massachusetts, USA (accepted for poster).
2. **Fan, C.**, He, J., 2011. Biotransformation of erythromycin by acclimated microorganisms in sequencing batch reactors (SBRs). Singapore International Water Week 2011. Singapore (accepted for presentation).

Chapter 1

Introduction

1.1 Background and problem statement

"Emerging contaminants are defined as compounds that are not currently covered by existing regulations of water quality, that have not been previously studied, and that are thought to be a possible threat to environmental health and safety" (Ferrer and Thurman, 2003). Based on this broad definition, emerging contaminants consist of diverse compounds, such as human and veterinary pharmaceuticals, personal care products, surfactants and surfactant residues, pesticide degradates, plasticizers, and various industrial additives (Ferrer and Thurman, 2003). It is only in recent years that the negative impacts of these contaminants on the environment have started to raise concern among the public, although most of these pollutants have been existent in the environment for decades. Thus, the concerns for these contaminants are emerging (Daughton, 2004). Nevertheless, the exact effects of many pollutants on humans and aquatic ecosystems are not well understood.

Antibiotics, one kind of emerging contaminants due to their potential to induce antibiotic resistant bacteria and transfer antibiotic resistance genes, have attracted growing attentions from researchers and the public over the past 20 years. Antibiotics initially originated from natural templates, which could be produced by particular species of bacteria or fungi as a competition mechanism to ensure their own survival (e.g., to gain a larger share of environmental substrate supplies by killing or inhibiting competitors) (Hancock, 2005). These natural antibiotics were firstly introduced into the clinical practice in the 1940s, and were proved efficient in dealing with diseases caused by pathogenic bacteria in human beings (Aminov, 2009). However, the emergence of multi-drug resistant pathogens has resulted in serious therapeutic difficulties in controlling infections using the natural antibiotics since the 1960s (Aminov, 2009). In order to cope with such super pathogens, tremendous amount of

money and time have been spent on modifying natural antibiotics to avoid resistance since the 1970s. Despite of such efforts, the exploitation of artificial antibiotics is still lagging behind the mutation of those super bugs. Therefore, efforts have shifted towards the control of usage and discharge of antibiotics in recent 20 years, because trace levels of antibiotics (e.g., from ng/L to μg/L levels) discharged into the environment by anthropogenic activities are suspected to select resistant bacteria and enhance resistance gene transfer in the environment. However, knowledge on correlation of antibiotic resistance development with antibiotics at environmental concentrations is still limited (Daughton and Ternes, 1999; Kummerer, 2009b).

Previously, it was assumed that antibiotics at lower environmental concentrations (from ng/L to μg/L levels) may play similar antibiotic roles, and develop similar resistance mechanisms in the same patterns as those at higher therapeutic concentrations (mg/L levels). However, it has been recently recognized that sub-inhibitory antibiotics are suspected to play signaling and regulatory roles in micro-ecosystems, while higher to lethal concentrations of antibiotics used in therapeutic practices act as a stress to inhibit or kill microorganisms (Davies et al., 2006; Linares et al., 2006; Martinez, 2008; Yim et al., 2006). The variability of antibiotic resistance genes in environmental bacteria are identified as a evolutionary result of ancient mutation for more than two billion years, but the rapid dissemination of resistance genes during the past 70 years are mainly due to the horizontal gene transfer among both taxonomically close and distant bacteria (Aminov, 2009). Previously, the mutation and gene transfer were considered as two parallel resistance gene development modes (Lipsitch and Samore, 2002). All the new understandings about roles of antibiotics and antibiotic resistance in nature have changed the current paradigm and driven us to clarify the relationship between antibiotic resistance and

sub-inhibitory antibiotics and to elucidate how resistance is regulated by low dose antibiotics in the environment.

The current occurrence of antibiotics in the environment is mainly from anthropogenic activities, such as wastewater discharge, manure disposal and aquaculture application (Kummerer, 2009a). Compared to the latter two, wastewater has a more direct influence on human beings due to the wide usage of recycled wastewater (e.g., as potable and non-potable water sources), which may spread many underused antibiotics to every spots of the world and may transfer antibiotic resistance genes to clinical pathogens (Ding and He, 2010; Le-Minh et al., 2010). However, studies about effects of antibiotics on wastewater are fewer compared to studies on manual-applied soil and antibiotics-contaminated aquaculture sediments. This is due to that: (1) relatively lower concentrations of antibiotics in wastewater may lead to less pronounced effects, (2) antibiotic resistant bacteria and resistance genes brought by wastewater may mask the antibiotic own effects on resistance development, and (3) mobile and lower density of microbes in wastewater may result in difficulty in detecting and comparing microbial community structures (Ding and He, 2010).

Fortunately, wastewater is finally collected in wastewater treatment plants (WWTPs), the compartments with higher diversity and density of microorganisms, in which the occurrence and transfer of new combination of resistance genes are found to be much more frequent (Murray, 1997). This has inspired researchers to investigate the occurrence of antibiotics and antibiotic resistance in WWTPs and downstream natural waters in the past decade (Le-Minh et al., 2010). However, investigations on causal relationship of antibiotics with intensification of resistance genes became increasingly difficult, because the exotic antibiotic resistance brought

by wastewater may mask the effects of antibiotics on resistance development, and there is a lack of reference WWTPs free from the input of resistance bacteria and genes. In addition, almost all current WWTPs have been contaminated with antibiotics. Without antibiotic-uncontaminated WWTPs as negative control, less pronounced influence of antibiotics on WWTPs performance, such as carbon, nitrogen and phosphorus removal, are difficult to be discovered due to lower concentrations of antibiotics. Moreover, since the majority of microorganisms in the environment (e.g. WWTPs) are not cultivated yet (Amann et al., 1995), high throughput uncultured-methods are necessary to detect, characterize and quantify both dominant and less dominant but important microbes in WWTPs. Otherwise, effects of low dose antibiotics on microbial community shift are unlikely to be discovered.

Among many kinds of antibiotics in WWTPs, erythromycin (ERY) and its derivative ERY-H_2O are among the antibiotics with the lowest removal rate in WWTPs (Rosal et al., 2010), and they are also among the most frequently detected antibiotics in surface water, ground water, and untreated drinking water sources (Focazio et al., 2008). Since ERY-H_2O is structurally similar to ERY, they both may have signaling functions as other sub-inhibitory antibiotics in the environment. Examples of the signaling functions include to stimulate horizontal gene transfer in microbial ecosystems, to select resistant bacteria among functionally redundant microorganisms, and to regulate microbial community components through cross-species talk (e.g., quorum-sensing (QS)). For instance, sub-inhibitory concentrations of ERY has been reported to activate the expression of specific gene encoding for polysaccharide intercellular adhesion in *Staphylococcus* (Rachid et al., 2000). Therefore, the influence of ERY and ERY-H_2O on the ecological function in WWTPs is worth studying.

1.2 Objectives and aims

In this study, we aim to investigate the effects of low concentrations of ERY (100 μg/L) and its derivative ERY-H_2O (50 μg/L) on ecological function disturbance (carbon, nitrogen and phosphorus removal), resistance selection and microbial community shift in lab-scale sequencing batch reactors (SBRs, the simulation of WWTPs).

Three SBRs (4L) were started up and operated over one year in exactly the same conditions, including seeding sludge, feeding synthetic wastewater (theoretical chemical oxygen demand (COD), NH_4^+–N, and PO_4^{3-}–P of 600, 60, and 15 mg/L, respectively), and an 8-hour operating batch mode, but differed only in terms of antibiotics spiked, ERY-H_2O of 50 μg/L (R1), ERY of 100 μg/L (R2), and no antibiotics (R3), respectively. Noteworthy, an 8-month pretreatment with the synthetic wastewater was applied on the seeding sludge in a mother reactor (MR) before being inoculated to the three SBRs. The pretreatment was expected to minimize residue antibiotics and antibiotic resistance, since the synthetic wastewater was absent of antibiotics, antibiotic resistance genes and resistant bacteria. Accordingly, the synthetic wastewater-feeding SBRs were free from input of exotic antibiotic resistance, and were able to demonstrate causal relationship of antibiotics with development of antibiotic resistance. In addition, R3 is used as an antibiotic-uncontaminated negative control reactor for comparison with another two reactors in terms of reactor performance and microbial community components. The specific scopes of studies are:

(1) To assess the influence of ERY or ERY-H_2O at low concentrations (μg/L) on the carbon, nitrogen, and phosphorus removal in SBRs. The inhibitory effects on

carbon and nutrients removal are evaluated by a long-term operation of the three SBRs. The nitrogen and phosphorus removal related microorganisms in the three steady state SBRs are analyzed by employing high-density phylogenic 16S rRNA gene microarrays (PhyloChip) containing 1,440 distinguishable prokaryotic operational taxonomic units (OTUs), and the community shifts in R1 (ERY-H_2O) and R2 (ERY) are compared with that in R3 (control). To verify whether the PhyloChip-observed nitrifying bacteria shifts are correlated with their resistance to ERY, short-term running batch experiments are conducted to study higher concentrations (100, 400, and 800 μg/L) of ERY's inhibition on nitrifying bacteria present in the biomass of the three steady state SBRs. The study will shed light on the influence of ERY and ERY-H_2O at the μg/l levels on the microbial ecological functions of treatment systems (e.g., the complex WWTPs as one of the most highly antibiotics-exposed environments).

(2) To identify the development of resistance genes and to investigate the biodegradation of ERY with microbial consortia acclimated to ERY (100 μg/L) or ERY-H_2O (50 μg/L) after long-term running with synthetic wastewater free from resistant bacteria and resistance genes input. Findings of this study will provide significant information on the inadequate data on effects of antibiotics to promote resistance gene development in the aquatic environment.

(3) To study influence of ERY or ERY-H_2O in the μg/l range on the microbial communities of the three SBRs. The 16S rRNA gene-based uncultured methods, including terminal restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE), and PhyloChip microarray, are used to statistically evaluate shift of microbial communities and identify selected and

inhibited microbial taxa. This study will substantiate the effects of low dose antibiotics to regulate microbes in WWTPs.

1.3 Organization of thesis

The thesis is subdivided into the following chapters, each defining a specific area of study that contributed to meeting the overall objective. Each chapter will contain individual introduction, materials and methods, results and discussion section specific to the area of study.

- **Chapter 2: Literature review**

  This chapter provides a comprehensive review of the history of antibiotics and antibiotic resistance, the role of antibiotics and antibiotic resistance in nature, the occurrence and fate of antibiotics in aquatic environments, the effects of antibiotics on ecological function disturbance, resistance selection and microbial community shift in aquatic environment.

- **Chapter 3: Influence of trace ERY and ERY-H_2O on carbon and nutrients removal and on resistance selection in SBRs**

  This chapter demonstrates low dose of ERY and ERY-H_2O affected the SBR performance in terms of carbon and nutrient removal via selection of resistant microorganisms.

- **Chapter 4: Proliferation of antibiotic resistance genes in microbial consortia of SBRs upon exposure to trace ERY or ERY-H_2O**

  This chapter exhibits the antibiotic resistance genes amplified by ERY and ERY-H_2O at low concentrations, and highlights the formation of antibiotic resistance biofilm as a result of ERY imposing on SBR microbial consortia.

- **Chapter 5: Decrease of bacterial diversity and enrichment of Betaproteobacteria in microbial consortia of SBRs exposed to trace ERY and ERY-H_2O**

  This chapter demonstrates the microbial community shift of SBRs due to sub-inhibitory ERY and ERY-H_2O.

- **Chapter 6: Conclusions and recommendations**

  The overall conclusion and recommendations for future studies are presented.

Chapter 2

Literature Review

2.1 The history of antibiotics and antibiotic resistance

The current knowledge on the antibiotic action modes and antibiotic resistance mechanisms is listed in Table 2.1. Different antibiotics have different working mechanism when attacking bacteria. The major targets of antibiotics include cell membranes (e.g., mupirocin), cell-wall biosynthesis enzymes and substrates (e.g., β-lactams, vancomycin, and bacitracin), bacterial protein synthesis (e.g., chloramphenicol, tetracyclines, macrolides, clindamycin, aminoglycosides, linezolid, mupirocin, and fusidic acid), and bacterial nucleic acid replication and repair (e.g., rifampicin, and quinolones) (Davies and Davies, 2010; Morar and Wright, 2010). According to the action modes of antibiotics, antibiotics can also be classified as bactericidal (causing death of bacteria) or bacteriostatic (preventing bacterial growth), which is not an intrinsic property of a given antibiotic but depends on the target species and/or the drug concentration (Hancock, 2005).

Mechanism of bacterial resistance to antibiotics can be defined in three main categories: the inactivation of the antibiotics by modification of its active chemical moiety (e.g., hydrolysis, phosphorylation, and glycosylation); the specific modification of the macromolecular target of antibiotics (e.g., by mutagenesis of key residues); and the prevention of antibiotics from reaching their targets through the excretion of antibiotic drugs via efflux pumps (Walsh, 2000). The variability of antibiotic resistance genes in environmental bacteria is currently indentified as an evolution result of ancient mutation for more than two billion years (Aminov, 2009). However, the past 70 year-propagation of antibiotic resistance genes is mainly due to the horizontal transfer of these genes across both taxonomically close and distant bacteria (Aminov, 2009).

Table 2.1 Modes of action and resistance mechanisms of antibiotics [a]

Antibiotic class	Antibiotics	Antibiotic target	Resistance mechanisms
β-Lactams	Penicillins (ampicillin), cephalosporins (cephamycin), penems (meropenem), monobactams (aztreonam)	Peptidoglycan biosynthesis	Hydrolysis, efflux, altered target
Aminoglycosides	Gentamicin, streptomycin, spectinomycin	Translation	Phosphorylation, acetylation, nucleotidylation, efflux, altered target
Glycopeptides	Vancomycin, teicoplanin	Peptidoglycan biosynthesis	Reprogramming peptidoglycan biosynthesis
Tetracyclines	Minocycline, tigecycline	Translation	Monooxygenation, efflux, altered target
Macrolides	Erythromycin, azithromicin	Translation	Hydrolysis, glycosylation, phosphorylation, efflux, altered target
Lincosamides	Clindamycin	Translation	Nucleotidylation, efflux, altered target
Streptogramins	Synercid	Translation	C-O lyase (type B streptogramins), acetylation (type A streptogramins), efflux, altered target
Oxazolidinones	Linezolid	Translation	Efflux, altered target
Phenicols	Chloramphenicol	Translation	Acetylation, efflux, altered target
Quinolones	Ciprofloxacin	DNA replication	Acetylation, efflux, altered target
Pyrimidines	Trimethoprim	C1 metabolism	Efflux, altered target
Rifamycins	Rifampin	Transcription	ADP-ribosylation, efflux, altered target
Lipopeptides	Daptomycin	Cell membrane	Altered target
Cationic peptides	Colistin	Cell membrane	Altered target, efflux

[a] The references (Davies and Davies, 2010; Morar and Wright, 2010)

The 70-year development of antibiotic resistance is nearly synchronous with the discovery of antibiotics, which is the same as other pharmaceuticals have suffered. The therapeutic usage of any pharmaceuticals is always coupled with the development of resistance to that drug. When sulfonamides, the first effective antibiotics, were applied for infectious treatment in 1937, the specific antibiotic resistance mechanism has been reported in several years later. In the 2010s, the therapeutic use of sulfonamides was overwhelmed (Davies and Davies, 2010). The synchronous history of antibiotics application in disease control and antibiotic resistance development can be divided into several decades as shown in Table 2.2.

The first era, named as the dark ages, is the period before 1940 when antibiotics had not been recognized by the people. The second era, namely the primordial era, is the decade in the 1940s when the chemotherapy initiated with the sulfonamide antibiotics, such as penicillin, streptomycin, and tetracycline. The third decade of the 1950s is named as the golden era because in this period large numbers of antibiotics were discovered and used till today, e.g., Kanamycin, erythromycin, vancomycin. In the fourth ten years of the 1960s, following the discovery of antibiotic fluoroquinolones, the amount of the new discovered antibiotics decreased, while the antibiotic resistant pathogens increased. Therefore, pharmacologist had to optimize the usage of these natural antibiotics to make up for the lack of new antibiotics. Therefore, the fourth era of the 1960s is called the pharmacologic era. In the later years, with the continuous increase of antibiotic resistant pathogens and the decrease of the discovery of natural antibiotics, people began to develop artificial antibiotics based on the understanding of the antibiotics and the resistance. The biochemical era is the period in the 1970s that artificial modification of the chemical structures of

antibiotics to avoid resistance were carried out relying on the knowledge of the biochemical actions of antibiotics and resistance mechanisms. In 1980s, the genetic studies of antibiotic targets led to design new compounds to avoid resistance. Therefore, the period is called the target era. In 1990s, the genomic screening methodology applied on pathogens was used to predict essential targets of antibiotics, and the high-throughput screening (HTS) assays on large amount of artificial antibiotics were used to select relevant antibiotics, which is called the Genomic HTS era. The following 2000s till now is called as the disenchantment era, since many companies disenchanted in the genome-based discovery programs on antibiotics because of the failure. In a word, the efforts to develop or modify antibiotics to avoid resistance have failed, because antibiotic resistance gene transferring across the entire biosphere occurred more easily and rapidly than the development of new antibiotics.

The development and distribution of antibiotic resistance genes among the microbial communities in the entire biosphere are believed to be accelerated by anthropogenic activities, such as the underuse, overuse, and misuse of antibiotics (Aminov, 2009; Davies and Davies, 2010). Therefore, efforts have been made to study the relationships between the low concentrations of antibiotics discharged by anthropogenic activities and the proliferation of antibiotic resistance genes. Before carrying out this investigation, it is also necessary to understand the roles of low concentrations of antibiotics in the natural ecosystems, as well as to recognize the development mechanisms of antibiotic resistance genes in the natural environments.

Table 2.2 History of antibiotic discovery and concomitant development of antibiotic resistance [a]

Time	Era name	Era description	Antibiotics discovered	Resistance
Pre-1940	Dark ages	Pre-antibiotic era		
1940s	Primordial	Advent of chemotherapy via the sulfonamides	Penicillin, streptomycin, tetracycline	
1950s	Golden	Most of the antibiotics used today were discovered	Kanamycin, erythromycin, vancomycin	
1960s	Pharmacologic	Attempts were made to understand and improve the use of antibiotics by dosing, administration, etc.	Fluoroquinolones, followed by a period of the low point of new antibiotic discovery and development	increasing
1970s	Biochemical	Knowledge of the biochemical actions of antibiotics and resistance mechanisms led to chemical modification studies to avoid resistance		
1980s	Target	Mode-of-action and genetic studies led to efforts to design new compounds		
1990s	Genomic HTS	Genome sequencing methodology was used to predict essential targets for incorporation into high-throughput screening (HTS) assays		
2000s	Disenchantment	With the failure of the enormous investment in genome-based methods, many companies discontinued their discovery programs	Synercid, linezolid	

[a] Adapted from the references (Davies and Davies, 2010; Wright, 2010)

2.2 The role of antibiotics and antibiotic resistance in nature

2.2.1 Updated knowledge on the roles of antibiotics and antibiotic resistance in nature

Since unavoidable antibiotic resistance always emerges rapidly after the anthropogenic usage of a drug, concerns on the roles of antibiotics and the origin and evolution of the antibiotic resistance in natural ecosystems are increasing. Previously, antibiotics were considered as a competition mechanism of antibiotics-producing bacteria or fungi to gain a larger share of environmental substrate supplies by killing competitors in their survival areas (Hancock, 2005). Therefore, higher to lethal concentrations of antibiotics were applied in the therapies to act as a stress to inhibit or kill microorganisms. Currently, investigations on the antibiotic resistance in environmental compartments have shed new light on the roles of antibiotics and antibiotic resistance in the nature. Different from therapeutically relevant concentrations of antibiotics that play roles to eliminate competitive microorganisms, the non-clinical or sub-inhibitory concentrations of antibiotics in the natural environment are recognized to play a universal signaling role in intra- and inter-domain communication in various ecosystems to select and survive adaptive phenotypic and genotypic microbes (Davies et al., 2006; Linares et al., 2006; Martinez, 2008; Yim et al., 2006). Therefore, the antibiotic resistance cannot be simply explained as the resistant mechanisms to the inhibitory or toxic effects of antibiotics. Instead, the antibiotic resistance can be generally defined as the universal microbial responses to the signaling effects of chemical compounds excreted by bacteria, fungi or more types of microbes. The signaling chemicals may include compounds other than the antibiotics known today. This may be one of the reasons to explain the phenomena in which antibiotic resistance genes have been found to be

persistent in the apparently antibiotic-free environments. The microbial responses to antibiotic signaling are identified in two categories as phenotypic responses and genotypic responses.

2.2.2 Antibiotic resistance roles – phenotypic responses to antibiotic signaling

It is not clear on the effective concentrations of antibiotics to induce cross-talk in natural environments. In lab-scale studies, however, bacterial responses to antibiotics were found to be concentration-dependent. Higher concentrations of antibiotics (mg/L levels) bring out a stress response, while lower concentrations of antibiotics (from ng/L to μg/L levels) regulate a specific set of genes in bacteria (Davies et al., 2006). These specific set of genes not only include those known to encode antibiotic resistance, but also include those encoding the transcriptional responses that may be converted into the phenotypes related to the pathogenic properties of bacteria.

Most of the bacteria that have been studied in labs on response to sub-inhibitory antibiotics are pathogens, among which the best-studied model is opportunistic pathogen *Pseudomonas aerugenosa.* One of the important pathogenic properties of *P. aerugenosa* is associated with the alginate overproduction and biofilm formation that are regulated by the quorum-sensing (QS) system. It has been reported that the low concentrations of antibiotics can regulate pathogenic properties of *P. aerugenosa* through QS system (Aminov, 2009). The lab studies on the responses of *P. aerugenosa* to sub-inhibitory concentrations of antibiotics, β-lactam antibiotics ceftazidime and imipenem, demonstrated contradictory effects of the two antibiotics on the decrease/increase alginate production and biofilm volume of *P. aerugenosa* (Aminov, 2009). This indicates that antibiotics even with the same molecular target

at their higher concentrations can result in conflicting impacts on alginate production, thereby suggesting that antibiotics have distinct action modes at sub-inhibitory and higher concentrations. In general, the factors that affect the regulation outcomes of low concentrations of antibiotics are complex and are largely unknown. Thus, the phenotypic responses of bacteria to sub-inhibitory concentrations of antibiotics need more investigation.

2.2.3 Antibiotic resistance roles – genotypic responses to antibiotic signaling

In contrast to phenotypic responses of bacteria to low concentrations of antibiotics that are inconclusive, genotypic responses are consistent in the conclusion that resistance gene transfer is highly possible to be enhanced by similar or unrelated antibiotics, and that low-dose antibiotics may result in the increase of mutation rates (Aminov, 2009). This may explain why antibiotic resistance genes exist in worldwide environment where low concentrations of antibiotics are detected. However, the correlation of input of antibiotics at lower environmental concentrations with the development or occurrence of antibiotic resistance genes is short of support with experimental data (Kummerer, 2009b). Other findings indicate that continuous input of resistant bacteria and resistance genes rather than the presence of antibiotics at sub-inhibitory concentrations may be more important for keeping resistance in the environment (Ohlsen et al., 2003; Ohlsen et al., 1998). Therefore, investigations are needed to clarify the causal effects of trace antibiotics on the development of antibiotic resistance genes in the aquatic and terrestrial environment.

On the other side, the new concept of antibiotic resistome has significantly expanded our understanding on the antibiotic resistance, and may help us to explicate the effects of sub-inhibitory concentrations of antibiotics on the proliferation of

antibiotic resistance. The antibiotic resistome is defined as a collection pool of all genes that contribute directly or indirectly to resistance, including four levels of genes as clinical, environmental, intrinsic and protoresistance (Wright, 2010). The interlock relationship of different levels of antibiotic resistance was shown in Fig. 2.1. The best-studied antibiotic resistance exists in the clinically important strains, which comprises a small part of genes associated with the resistance. The second level of antibiotic resistance is distributed in a large amount of environmental bacteria, especially antibiotic producers. The environmental resistome are more diverse than the clinical resistome, and serve as a reservoir of the clinical resistome. The third level of resistance is the intrinsic resistance contained by bacteria, such as efflux systems prevalent on gram-negative bacteria can also perform as efflux pumps of antibiotics. The most broad and higher level of resistance is the protoresistome, which encode metabolic proteins unrelated or a bit related to antibiotic resistance, but that can be evolved into resistance genes. The protoresistome serves as a pool of precursors of antibiotic resistance. The resistome describes the multiple sources of antibiotic resistance widely distributed in the environments, and may explain why resistance emerges so rapidly after antibiotic application in the clinic pathogens. Moreover, the resistance gene-containing strains could have high fitness in the antibiotics-absent environments, therefore, resistance genes are not easily lost and continue to persist when antibiotic pressure is not present (Andersson, 2006; Enne et al., 2004; Enne et al., 2005; Lenski, 1997; Luo et al., 2005). However, it is largely unknown how the antibiotics-regulated cross-talk occurs in natural environments. The knowledge on the influence of antibiotics on the microorganisms in the environments is limited.

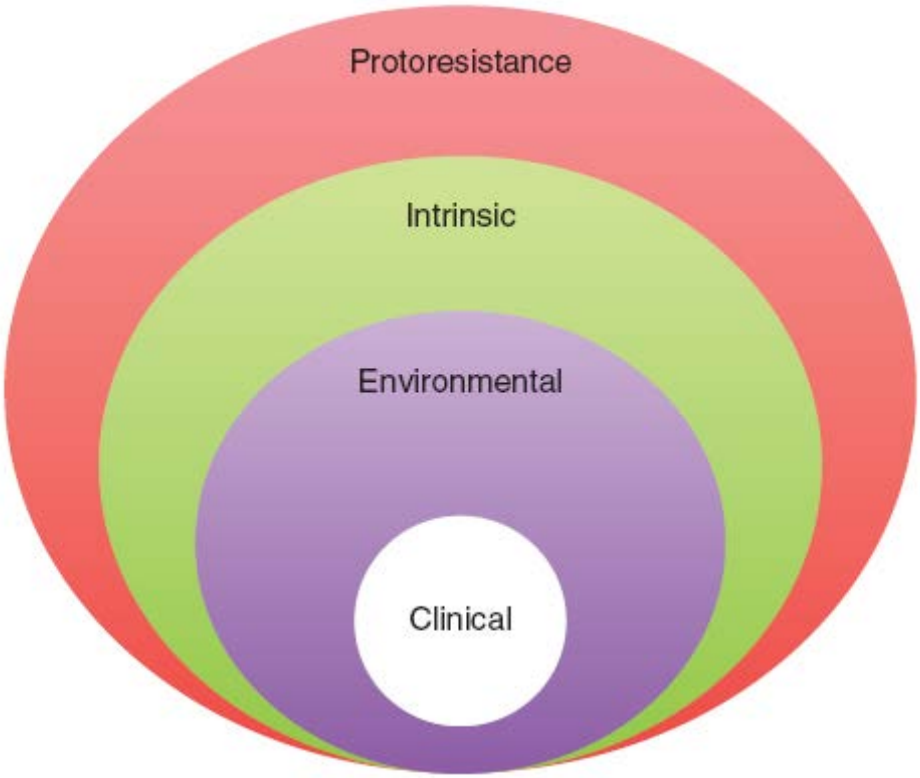


Fig. 2.1 The antibiotic resistome (Wright, 2010)

2.3 The occurrence and fate of antibiotics in aquatic environment, especially in sewage treatment processes

2.3.1 The origins and dissemination of antibiotics in the environment

Most antibiotics are naturally produced by the microorganisms in the soil. The concentrations of these antibiotics in the soil are dependent on the density of the antibiotic producers in the environment. In aquatic environment, the density of microorganisms is lower than that in the soil; thereby the concentrations of these naturally-produced antibiotics in these compartments may be even lower. Up to now, there is no report on the concentrations of antibiotics naturally produced in the aquatic environment. It is believed that the main source of pharmaceuticals, including antibiotics in the environment, is the anthropogenic input (Kummerer, 2009a). The

massive production and application of antibiotics in recent years greatly enhance the dissemination of antibiotics into every part of the environments.

Once antibiotics are produced in the factories, they begin to contact directly/indirectly with human beings (Fig.2.2). Besides the intentional disposal of precursors, byproducts, and unused drugs of antibiotics into sewage and soil, the antibiotics present in the environment are largely due to human, animals and aquaculture excretion, as well as agriculture applications. Not all active antibiotics are completely metabolized during therapeutic use, and then both unchanged and metabolized drugs enter sewage and soil (Hirsch et al., 1999; Kummerer, 2009a). In sewage treatment plants, since most antibiotics are not readily degradable, the antibiotics and their derivatives cannot be completely removed from liquid sewage through conventional processes of WWTPs. Even the antibiotics adsorbed to the solid activated sludge of WWTPs will be excreted into sewage effluent again during the period of excess sludge digestion (Le-Minh et al., 2010). Therefore, untreated antibiotics and their structure-close derivatives may subsequently enter into surface water, further run into the sources of drinking water, and lead to the increase of the occurrence of antibiotic resistance genes and resistance gene-containing bacteria. Similarly, unused antibiotics and their derivatives in manure, sediments and digested activated sludge are finally applied on soil as the manure. The runoff of rain and irrigation may play crucial roles to disseminate these chemicals into surface water, ground water and water source. Whatever routes the antibiotics and their derivatives have transferred, these compounds are persistent in the environment. Therefore, these persistent antibiotics may be recycled to contact with people, animal and plants again. The long-term effect would be that the antibiotic resistance maintains in certain levels in the environment due to the presence of antibiotics and their derivatives.

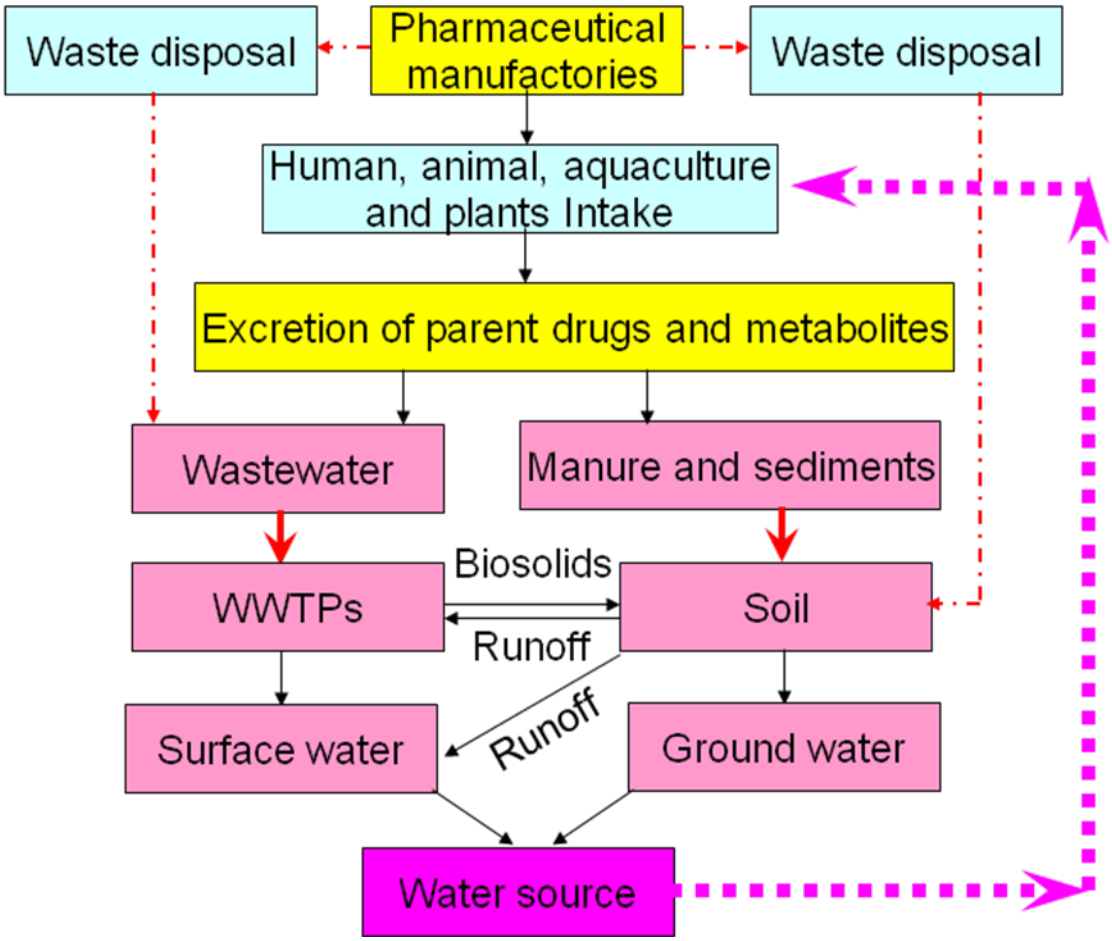


Fig. 2.2 Origins and dissemination of antibiotics in the environment. Summarized according to the reference (Kummerer, 2009a).

2.3.2 The occurrence and fate of antibiotics in conventional WWTPs and downstream receiving water bodies

As the main collection pools of antibiotics and antibiotic resistant bacteria, WWTPs are posing increasing threat to human beings because of the wide usage of the recycled wastewater (e.g., as portable and non-portable water sources) (Le-Minh et al., 2010). The occurrence levels of antibiotics in raw sewage, effluent of coventioanl WWTPs, and downstrean reveiving water bodies (e.g., surface water and ground water) are summarized in Table 2.3.

The detected concentrations of specific antibiotics in raw sewage differed between countries, possibly reflecting variable prescription practices, as well as the

various degrees of dilution due to differences in per-capita water consumption (Le-Minh et al., 2010; Miao et al., 2004). For example, since sulfamethoxazole is one of the top 15 pharmaceuticals sold in China, its concentrations were reported as high as 7.91 µg/L in sewage influent (Peng et al., 2006). For macrolides, clarithromycin is more often detected in WWTPs effluents at higher concentrations than erythromycin-H_2O and roxythromycin in Switzerland, whereas erythromycin-H_2O is more frequently detected than clarithromycin and roxythromycin in Canada, which is well correlated to consumption pattern (Gobel et al., 2007; Miao et al., 2004).

The removal efficiency of antibiotics in conventional WWTPs is dependant on both the properties of antibiotics and the processes of WWTPs. Through hydrophobic interactions with organic-rich sewage sludge, hydrophobic antibiotics can be removed from liquid phase more efficiently than hydrophilic chemicals (Beausse, 2004). Antibiotics can also be transferred from aqueous solution onto solid sludge via ion exchange, complex formation with metal ions, and polar hydrophilic interaction (Diaz-Cruz et al., 2003). For example, macrolides are removed mainly by sorption to sewage sludge in WWTPs via hydrophobic interactions and cation exchanges due to its surfactant-like structure, and their average removal efficiency can be up to 50% for clarithromycin and azithromycin (Yasojima et al., 2006). Fluoroquinolones are predominantly removed by adsorption to sludge, and their removal efficiency is reported to be 88–92% (Golet et al., 2003).

Besides the above mentioned sorption mechanisms, antibiotics can also be transformed by hydrolysis and further biotransformation in WWTPs. For example, the cyclic amide of all β-lactam drugs is highly susceptible to be hydrolyzed chemically or enzymatically (Deshpande et al., 2004). The chemical hydrolysis of β-lactam penicillin can occur with acid, alkaline, and even nucleophiles (water or metal

ions). The β –lactamases are the enzymes contained by many species of bacteria to hydrolyze β-lactams. In WWTPs, β-lactams can be significantly bio-degraded through biological processes, and β-lactams are rarely detected in the effluent of WWTPs (Watkinson et al., 2007). Besides β-lactams, sulfonamides that are adsorbed to the sludge can be effectively removed by biodegradation in the process of anaerobic digestion (Carballa et al., 2006).

The biotransformation of antibiotics are significantly affected by operating conditions of WWTPs (Beausse, 2004). For example, the increase of solids retention time (SRT) and hydraulic retention time (HRT) are expected to provide sufficient enrichment and reaction time for slower growing bacteria, which may be capable of transforming antibiotics (Clara et al., 2005; Jones et al., 2007; Kim et al., 2005; Tauxe-Wuersch et al., 2005; Vieno et al., 2007; Yasojima et al., 2006). Removal efficiency of trimethoprim can be improved in WWTPs with longer SRT, because nitrification micriorganisms have been reported to degrade trimethoprim (Batt et al., 2006; 2007; Perez et al., 2005). Moreover, SRT is also a factor to affect the biosorption of antibiotics, because the change of activated sludge characters may influece the biosorption ability of sludge. Shorter SRT has been reported to significantly reduce the removal efficiency of tetracycline (Kim et al., 2005).

In summary, conventional WWTPs processes appear to be variable in removing antibiotics, and antibiotics adsorbed to activated sludge are expected to be released into liquid phase during the processes of anaerobic sludge digestions. As a result, antibiotics are detected in downstream surface water and ground water, where the antibiotic resistance genes is also expected to be proliferated due to the presence of antibiotics (Adelowo et al., 2008).

Table 2.3 Measured concentrations of antibiotics in the water environments [a]

Group /Antibiotics	IF (ng/l)	EF (ng/l)	Removal efficiency (%)	Main process of WWTPs	SW (ng/l)	GW (ng/l)	References
β-lactam							
Cephalexin	2000	80	96	Activated sludge system (AS)			(Costanzo et al., 2005)
	≤5600	<(2)	–	AS			(Watkinson et al., 2007)
	670–2900	240–1800	9– 89	Chemical enhanced / Secondary treatment			(Gulkowska et al., 2008)
	1563–4367	10–994	36–99.8	Secondary treatments/UV or chlorination			(Lin et al., 2009)
Amoxicillin	≤280	<(3) 30	–	AS			(Watkinson et al., 2007)
Cloxacillin	<(1)–320	<(1)	–	AS			(Watkinson et al., 2007)
	<(13)–15	<(9)	–	Secondary treatment / Chlorination			(Cha et al., 2006)
Penicillin G	<(2)	<(2)	–	AS	up to 3		(Watkinson et al., 2007; Färber, 2002)
Penicillin V	≤160	≤80	–	AS	up to 3		(Watkinson et al., 2007; Färber, 2002)
Flucloxacillin					7		(Christian et al., 2003)
Piperacillin					48		(Christian et al., 2003)
Sulfonamides							
Sulfamethoxazole	1090	210	81	AS/chlorination	up to 163	up to 410	(Yang et al., 2005; Sacher et al. 2002)
	450	<(30)	>93	AS	up to 480	up to 470	(Choi et al., 2007; Hirsch et al., 1999)
	5450–7910	<100	>98	AS/filtration/chlorination	up to 52		(Christian et al., 2003; Peng et al., 2006)
	590	0.39	33	AS	up to 1900		(Gros et al., 2006; Kolpin et al., 2002)
	230–570	210–860	–	AS/Sand filtration			(Gobel et al., 2005)
	390	0.31	20	AS			(Brown et al., 2006)
	<(50)–1250	<(50)–210	18–100	AS			(Bendz et al., 2005; Karthikeyan and Meyer, 2006)
	20	70	–	AS/Chemical P removal			(Bendz et al., 2005)

Group /Antibiotics	IF (ng/l)	EF (ng/l)	Removal efficiency (%)	Main process of WWTPs	SW (ng/l)	GW (ng/l)	References
	580	250	67	AS			(Carballa et al., 2004)
	0.6	NA	57	AS			(Carballa et al., 2005)
	<(80)–674	<(80)–304	42	Chemical P removal/AS			(Lindberg et al., 2005)
	24–145	18–91	– 279 to 66	AS			(Clara et al., 2005)
	10–118	9–78	34–63	AS or Chemical enhanced / UV or chlorination			(Xu et al., 2007a)
	179–1760	47–964	26–88	Secondary treatments/UV or chlorination			(Lin et al., 2009)
N4-sulfamethoxazole	850–1600	<(20)–180	–	AS/Sand filtration			(Gobel et al., 2005)
Sulfathiazole	10 570	180	98	AS	up to 130		(Choi et al., 2007; Kolpin et al., 2002)
Sulfamethazine	150	<(30)	>80	AS/chlorination		up to 160	(Hirsch et al., 1999; Yang et al., 2005)
	4010	<(30)	>99	AS	up to 220		(Choi et al., 2007; Kolpin et al., 2002)
Sulfadimethoxine	70	<(30)	>57	AS/chlorination	up to 60		(Kolpin et al., 2002; Yang et al., 2005)
	460	<(30)	>93	AS			(Choi et al., 2007)
Sulfadiazine	5100–5150	<(150)	> 97	AS/filtration/chlorination		up to 17	(Peng et al., 2006; Sacher et al. 2002)
	<(1)–72	<(1)–36	50	AS or Chemical enhanced/UV or chlorination			(Xu et al., 2007a)
Sulfamerazine	1530	< (30)	> 98	AS			(Choi et al., 2007)
Sulfadimidin						up to 23	(Sacher et al., 2002)
					up to 7		(Christian et al., 2003)
Trimethoprim							
Trimethoprim	1172	290	75	AS	up to 24		(Gros et al., 2006; Sacher et al., 2002)
	210–440	20–310	64	AS	up to 200		(Gobel et al., 2005; Hirsch et al., 1999)
	0.59	180	70	AS	up to 12		(Brown et al., 2006; Christian et al., 2003)
	0.14–1.10	<(50)–550	50– 100	AS	up to 710		(Karthikeyan and Meyer, 2006; Kolpin et al., 2002)

Group /Antibiotics	IF (ng/l)	EF (ng/l)	Removal efficiency (%)	Main process of WWTPs	SW (ng/l)	GW (ng/l)	References
	80	40	49	AS/chemical P removal			(Bendz et al., 2005)
	213–300	218–322	– 3	Trickling filter/AS/UV			(Roberts and Thomas, 2006)
	99–1300	66–1340	3	Chemical P removal/AS			(Lindberg et al., 2005)
	120–320	120–230	– 17 to 62	Chemical enhanced / Secondary treatment			(Gulkowska et al., 2008)
	259–949	203–415	22–56	Secondary treatments/UV or chlorination			(Lin et al., 2009)
Tetracyclines							
Doxycycline	210	70	67	AS/chlorination			(Yang et al., 2005)
	220	30	86	AS			(Choi et al., 2007)
	<(64)–2480	<(64)–915	70	Chemical P removal/AS			(Lindberg et al., 2005)
Tetracycline	200	< (30)	>85	AS/chlorination	up to 110		(Kolpin et al., 2002; Yang et al., 2005)
	110	<(0.03)	>73	AS			(Choi et al., 2007)
	240–790	<(50)–160	68–100	AS			(Karthikeyan and Meyer, 2006)
	96–1300	180–620	– 88 to 73	Chemical enhanced / Secondary treatment			(Gulkowska et al., 2008)
	46–234	16–38	66–90	Secondary treatments/UV or chlorination			(Lin et al., 2009)
Chlortetracycline	270	60	78	AS/chlorination	up to 690		(Kolpin et al., 2002; Yang et al., 2005)
	970	40	96	AS			(Choi et al., 2007)
Oxytetracycline	240	<(30)	>88	AS	up to 340		(Choi et al., 2007; Kolpin et al., 2002)
					up to 19.2		(Calamari et al., 2003)
Chinolones							
Ciprofloxacin		up to 100			up to 5		(Färber, 2002)
	<(50)–310	<(50)–60	22–100	AS	9		(Christian et al., 2003; Karthikeyan and Meyer, 2006)

Group /Antibiotics	IF (ng/l)	EF (ng/l)	Removal efficiency (%)	Main process of WWTPs	SW (ng/l)	GW (ng/l)	References
	90–300	<(6)–60	87	Chemical P removal/AS	up to 30		(Kolpin et al., 2002; Lindberg et al., 2005)
	90	130	– 44	AS	up to 26.2		(Calamari et al., 2003; Costanzo et al., 2005)
	320–570	60–90	83	AS/Fe flocculation			(Golet et al., 2003)
	320	31.5	90	AS/Chemical P removal/sand filtration			(Zorita et al., 2009)
	80	27	66	Secondary treatment			(Xiao et al., 2008)
Norfloxacin	340–520	40–60	88	Act. Sludge/Fe flocculation	up to 120		(Golet et al., 2003; Kolpin et al., 2002)
	66–174	<(7)–37	87	Chemical P removal/AS			(Lindberg et al., 2005)
	18	<(5.5)	>70	AS/Chemical P removal/sand filtration			(Zorita et al., 2009)
	110–460	85–320	– 20 to 78	Chemical enhanced/Secondary treatment			(Gulkowska et al., 2008)
	54–263	27–85	50–82	AS or Chemical enhanced/UV or chlorination			(Xu et al., 2007a)
	339	85	75	Secondary treatment			(Xiao et al., 2008)
Ofloxacin	<(43)	<(43)	–	AS			(Gros et al., 2006)
	3520–5560	<(80)–740	>85	AS/filtration/chlorination	20		(Alexy et al., 2006; Peng et al., 2006)
	470	110	77	AS			(Brown et al., 2006)
	<(6)–287	<(6)–45	86	Chemical P removal/AS			(Lindberg et al., 2005)
	22.5	10	56	AS/Chemical P removal/sand filtration			(Zorita et al., 2009)
	80–368	41–165	40– 70	AS or Chemical enhanced/UV or chlorination			(Xu et al., 2007a)
	115–1274	53–991	<88	Secondary treatment/UV or chlorination			(Lin et al., 2009)
	1208	503	58	Secondary treatment			(Xiao et al., 2008)

Group /Antibiotics	IF (ng/l)	EF (ng/l)	Removal efficiency (%)	Main process of WWTPs	SW (ng/l)	GW (ng/l)	References
Macrolides							
Erythromycin	<(20)	<(20)	–	AS		up to 49	(Gros et al., 2006; Sacher et al., 2002)
	60–190	60–110	–	AS	up to 1700		(Gobel et al., 2005; Hirsch et al., 1999)
	<(50)–1200	<(50)–300	44–100	AS or aerated lagoon	up to 190		(Alexy et al., 2006; Karthikeyan and Meyer, 2006)
	71–141	145–290	– 79	Trickling filter/Act. sludge/UV	up to 1700		(Kolpin et al., 2002; Roberts and Thomas, 2006)
	470–810	520–850	– 12 to 19	Chemical enhanced/Secondary treatment	up to 15.9		(Calamari et al., 2003; Gulkowska et al., 2008)
	253–1978	216–2054	15–45	AS or Chemical enhanced/UV or chlorination			(Xu et al., 2007a)
	226–1537	361–811	<56	Secondary treatments/UV or chlorination			(Lin et al., 2009)
Roxithromycin	10–40	10–30	–	AS/sand filtration		up to 26	(Gobel et al., 2005; Sacher et al., 2002)
	1500	870	42	AS or aerated lagoon	up to 560		(Hirsch et al., 1999; Karthikeyan and Meyer, 2006)
	25–117	36–69	– 80 to 44	AS	up to 14		(Alexy et al., 2006; Clara et al., 2005)
	75–164	35–278	53–76	AS or Chemical enhanced/UV or chlorination	up to 180		(Kolpin et al., 2002; Xu et al., 2007a)
Clarithromycin	330–600	110–350	21	AS/sand filtration	up to 65		(Giger et al., 2003; Gobel et al., 2005)
	492–883	266–444	43	AS	up to 260		(Hirsch et al., 1999; Yasojima et al., 2006)
	59–1433	12–232	<0 to 99	Secondary treatments/UV or chlorination	up to 37		(Alexy et al., 2006; Lin et al., 2009)
					up to 20.3		(Calamari et al., 2003)
Azithromycin	199–371	88–219	49	AS	up to 3		(Alexy et al., 2006; Yasojima et al., 2006)
Others							

Group /Antibiotics	IF (ng/l)	EF (ng/l)	Removal efficiency (%)	Main process of WWTPs	SW (ng/l)	GW (ng/l)	References
Ronidazol						up to 10	(Sacher et al., 2002)
Chloramphenicol		up to 68					(Christian et al., 2003)
		up to 560			up to 60		(Hirsch et al., 1999)
Clindamycin		up to 110					(Christian et al., 2003)
					up to 24		(Alexy et al., 2006)
Lincomycin					up to 730		(Kolpin et al., 2002)
					up to 248.9		(Calamari et al., 2003)
Spiramicin					up to 74.2		(Calamari et al., 2003)
Oleandomycin					up to 2.8		(Calamari et al., 2003)
Tylosin					up to 280		(Kolpin et al., 2002)
					up to 2.8		(Calamari et al., 2003)

[a] Summarized from the references (Kummerer, 2009a; Le-Minh et al., 2010)
Value in the parenthesis is the limit of detection in each study. AS: activated sludge
Abbreviations: IF, EF influent and effluent water of sewage treatment plant, SW surface water, GW ground water

2.4 The effects of antibiotics on ecological function disturbance, resistance selection and microbial community shift in aquatic environment

Most of antibiotics and their metabolites are persistent in the environments, even in the manually enhanced biological treatment processes of WWTPs. The persistent antibiotics and derivatives are believed to play roles as ecological factors that could induce phylogenetic structure alteration, resistance expansion, and ecological function disturbance of environmental ecology (Ding and He, 2010). Compared to soil compartments, however, fewer studies have been conducted on WWTPs. In WWTPs, the lower concentrations of antibiotics, higher mobility of microbes, and the masking of larger quantities of antibiotic resistance genes and resistant bacteria input into the aquatic environment have resulted in less pronounced effects of antibiotics being discovered hardly (Ding and He, 2010). Since signaling and regulatory roles of sub-inhibitory antibiotics on microbes have been recognized recently, studies of antibiotic influence on WWTPs were rarely related to the contents, which are not necessarily weaker in aquatic ecosystem than in soil. Therefore, in order to shed light on microbial responses to antibiotic signaling, it is imperative to study the influence of low-dose antibiotics on complex microbial consortia of WWTPs, in terms of bacterial performance interruption, microbial community shift, and antibiotic resistance proliferation.

2.4.1 The effects of antibiotics on ecological function disturbance and on microbial community shift in aquatic environment

Antibiotics are frequently detected in sewage in the concentrations of ng/L and μg/L ranges (Le-Minh et al., 2010). These concentrations are not considered to be high enough to cause noticeable effects on wastewater treatment processes. However,

the higher concentrations (mg/L ranges) of antibiotics have been reported to impose inhibitory impacts on the microbial activities in WWTPs. The sulfonamide antibiotics (10–400 mg/L) have been reported to inhibit microbial activities in activated sludge by more than 20% (Ingerslev and Halling-Sorensen, 2000). Nitrogen transformation process has been best-studied. Streptomycin at 400 mg/L can achieve 75% inhibition on ammonia oxidation in activated sludge (Tomlinso.Tg et al., 1966). Oxytetracycline has shown to inhibit nitrification process in surface water (Klaver and Matthews, 1994). In anaerobic processes, erythromycin of 1 mg/L has been reported to reduce COD removal efficiency and biogas production by about 5% (Amin et al., 2006). Metronidazole (6 mg/L) could reduce anaerobic activity by 69% (Gartiser et al., 2007a). Moreover, methanogenesis has been reported to be inhibited by antibiotics sulfamethoxazole and ofloxacin in an anaerobic digestion process (Fountoulakis et al., 2004). Ofloxacin has been found to inhibit both processes of methanogenesis and acetogenesis (Fountoulakis et al., 2008). In contrast, even with longer reaction time up to 80 h, ampicillin (250 mg/L), benzylpenicillin (250 mg/L), novobiocin (150 mg/L), oxytetracycline (250 mg/L), and chloramphenicol (50 mg/L) have shown no effect on either biomass or nitrate production in a stabilized nitrifying sludge (Gomez et al., 1996). In some cases when a complex mixture of bacteria has been exposed to antibiotics, however, increased nitrification activity can be observed with the reason not yet clear (Halling-Sorensen, 2001).

Then what are the responses of microorganism to antibiotics at low concentrations? The microbial community structures of WWTPs are highly possible to shift in the way that antibiotic sensitive bacteria are substituted by resistant bacteria, which may be intrinsic resistance or achieve resistance genes via horizontal gene transfer due to antibiotic pressure (Ding and He, 2010; Szczepanowski et al., 2009).

For example, tetracycline resistant bacteria have been shown to increase in sequencing batch reactor exposure to tetracycline of 1 mg/L (Kim et al., 2007). Bacteria resistant to ciprofloxacin, trimethoprim, sulfamethoxazole and vancomycin have been found in wastewater influent and effluent, such as fecal coliforms, *E.coli* and *enterococci* (Nagulapally et al., 2009). Most importantly, resistant bacteria are highly possible to play similar ecological functions as the substituted sensitive bacteria in the complex systems, although they belong to same or distinct species (Ding and He, 2010). Consequently, the selection of resistant bacteria can offset the inhibitory impact of antibiotics on the functional bacteria associated with the performance of WWTPs. Nevertheless, it is still inconclusive on the actual significance of low concentrations of antibiotics in WWTPs in term of resistance propagation. Therefore, it is necessary to study the effects of low levels of antibiotics in WWTPs on the selection of resistance genes and resistant bacteria. Further study is also needed to investigate the microbial community structure shift due to the presence of antibiotics at low concentrations.

2.4.2 The effects of antibiotics on resistance selection in aquatic environment

It is still under debate which factor is more important for the proliferation of antibiotic resistance in WWTPs, e.g., the input of resistant bacteria and resistance genes with wastewater, or the presence of ultra-low concentrations of antibiotics in wastewater. However, it is consistent that massive antibiotic resistance genes have been detected in untreated sewage (US), activated sludge (AS) and effluent of WWTPs (EW), special wastewater (SW) from hospital, animal production, and aquaculture area, natural water (NW), sediments (SD) and drinking water (DW) as shown in Table 2.4.

The antibiotic resistance genes detected in the WWTPs include those resistant to all kinds of antibiotics used today, e.g., aminoglycoside, β-lactam, chloramphenicol, fluoroquinolone, macrolide, rifampicin, tetracycline, trimethoprim and sulfonamide, as well as multidrug efflux genes and small multidrug resistance genes (Szczepanowski et al., 2009; Zhang et al., 2009). Also, the spectra of antibiotic resistance genes detected in activated-sludge and in effluent of sewage are quite similar. The same genes resistant to fluoroquinolone, trimethoprim and sulfonamide, as well as the same genes for multidrug efflux systems have been detected in both activated sludge and the effluent (Szczepanowski et al., 2009). The only difference is that the detected numbers of genes resistant to aminoglycoside, β-lactam, macrolide and tetracycline are slightly less in final effluent than in the activated sludge (Szczepanowski et al., 2009). This indicates that sewage treatment processes used in the WWTPs are not effective to remove antibiotic resistance genes, especially fluoroquinolones, trimethoprim and sulfonamides resistance genes. The persistence of antibiotic resistance genes is consistent with the persistence of antibiotics in the WWTPs. Fluoroquinolones are predominantly removed by adsorption to sludge (Golet et al., 2003). Trimethoprim are negligible to be removed by sorption to activated sludge; only in the WWTPs with longer SRT that allow nitrification microorganisms to sustain, biodegradation efficiency of trimethoprim by nitrifiers can be improved (Batt et al., 2006; 2007; Perez et al., 2005). Sulfonamides that are sorbed to the sludge can be effectively removed by biodegradation in the process of anaerobic digestion, but not in the aerobic processes of WWTPs (Carballa et al., 2006). The presence of these antibiotics in activated sludge and in liquid sewage might exert selective pressure on bacteria within the sewage treatment system, leading to the enrichment of resistant bacteria/genes and their release into the environment

with the final effluents. This is proved by the fact that antibiotic resistance genes can also be detected in bacteria obtained from the downstream WWTP, such as natural water and even drinking water, indicating that these resistance genes have been further dispersed downstream WWTPs (Zhang et al., 2009).

Moreover, some recently discovered resistance genes from clinical isolates were also detected in WWTPs, indicating exchanges of resistance genes between clinical and WWTPs bacteria. These newly indentified resistance genes from clinical strains that are also detected in WWTPs include the aminoglycoside resistance genes *aadA6/aadA10* and *aac(3)-Id*, the β-lactam resistance genes *ctx-m-27*, *ctx-m-32*, *ges-3*, *imp-9*, *imp-13* and *oxa- 58*, and the fluoroquinolone resistance genes *qnrA3*, *qnrB1* and *qnrS* (Szczepanowski et al., 2009). The results suggest that WWTP bacteria are a reservoir for various resistance genes that are collected from clinical sources. The clinically relevant resistant bacteria and genes may be further disseminated to natural environments. Therefore, it is crucial to clarify how these resistance genes, under the selection of low levels of antibiotics, could transfer among bacteria and alter the microbial communities of WWTPs in order to maintain the level of resistance.

Table 2.4 Antibiotic resistance genes detected in the water environments [a]

Gene name*	**Gene product**	**Resistance to**	**Detected in water environments**
aacA, aadB	Aminoglycoside 6'-N-acetyltransferase	Km, Tob, Ak	AS,EW
aacA1	Aminoglycoside 6'-N-acetyltransferase	Gm, Km, Tob, Neo	AS
aacA4	Aminoglycoside 6'-acetyltransferase	Ak	AS, NW
aacA7	Aminoglycoside acetyltransferase-6' type I	Gm, Tob, Km	AS
aacA29b	Aminoglycoside 6'-N-acetyltransferase	Ak, Km	AS, EW
aacC1	Aminoglycoside 3N-acetyltransferase	Gm	AS, EW,NW, SW, US
aacC2	Aminoglycoside (3)-N-acetyltransferase	Gm	AS, EW, NW, SW, US
aacC3	Aminoglycoside (3)-N-acetyltransferase	Gm	NW, SW, US
aacC4	Aminoglycoside (3)-acetyltransferase IV	Gm	AS, EW, NW, SW, US
*aac(3)-Id**	3'-N-Aminoglycoside acetyltransferase	Gm	AS, EW
aac(69)-Im	6'-Aminoglycoside N-acetyltransferase	Tob, Ak, Km	AS, EW
aadA4, aadA5	Streptomycin 3'-adenylyltransferase	Sm, Sp	AS, NW
aadA7	Aminoglycoside (3')(9)-adenylyltransferase	Sm, Sp	AS, EW
aadA9	Streptomycin 3'-adenylyltransferase	Sm, Sp	AS
*aadA10, aadA6/aadA10**	Aminoglycoside (3')(9)-adenylyltransferase	Sm, Sp	AS, EW
aadA12, aadA1, aadA2, aadA8, aadA11, aadA13, aadA23	Putative streptomycin 3'-adenylyltransferase	Putative Sm, Sp	AS, DW, EW, NW, SD, SW, US
aadD	Kanamycin-nucleotidyltransferase	Km	AS, EW
aph	Aminoglycoside 3'-phosphotransferase	Km, Neo	AS, EW
aphA	3'-Aminoglycoside phosphotransferase	Km	DW
aphA-3	3'5'-Aminoglycoside phosphotransferase of type III	Km	AS, EW
aphA-6	3'-Aminoglycoside phosphotransferase	Km, Ak	AS, EW
aph2	Aminoglycoside-3'-O-phosphotransferase	Km, Neo	AS, EW
aph(29)-Ib	Aminoglycoside phosphotransferase	Km	AS
strA	Aminoglycoside 3'-phosphotransferase	Sm	AS, NW, SW
strB	Aminoglycoside 6-phosphotransferase	Sm	AS, NW
ctx-m-4	Class A β-lactamase	Amp, Ctx, Cxm, Atm	AS, EW
*ctx-m-27**	Class A β-lactamase	Caz, Ctx, Amo, Tic, Prl, Kf, Cxm, Cpo, Atm	AS, EW
*ctx-m-32**	Class A β-lactamase	Amo, Ctx, Caz, Fep, Prl, Kf, Fox, Cxm	AS, EW

*Gene name**	Gene product	Resistance to	Detected in water environments
*ges-3**	Class A extended-spectrum β-lactamase	Titeracillin, Prl, Caz, Ctx, Atm, Ipm	AS, EW
per-2	Class A extended-spectrum β-lactamase	Oxyiminocephalosporins, Atm, Cft	AS
shv-34	Class A β-lactamase	Caz, Ctx	AS, EW
blaTEM-1	Class A β-lactamase	Amp, Pen-G	AS, EW
blaTLA-2	Class A extended spectrum β-lactamase	Amo, Tic, Caz, Kf, Cxm, Fox, Ctx, Fep, Atm	AS, EW
veb-1	Class A extended-spectrum β-lactamase	Cephalosporins, Atm	AS
vim-4	Metallo-β-lactamase	β-lactamase	EW
imp-2, imp-5	Class B metallo β-lactamase	Amp, Ctx, Fep	AS, EW
imp-9, imp-11*	Class B metallo β-lactamase	β-lactamase	AS, EW
imp-13, imp-2	Class B metallo β-lactamase	Cxm, Caz, Ctx, Cro, Fep, Amp	AS, EW
ampC	Class C β-lactamase, cephalosporinase	Pen, cephalosporins	DW, NW, SW, US
cmy-9, cmy-10	Class C β-lactamase	β-lactamase	AS, EW
cmy-13, cmy-5*	Class C β-lactamase	β-lactamase	AS, EW
blaNPS-1	Class D β-lactamase	Amo, azlocillin, Cec, cefazolin, Cfp, Prl	AS
blaNPS-2	Class D β-lactamase	Amp	AS, EW
oxa-1	Class D β-lactamase	β-lactamase	AS, EW
oxa-2, oxa-21, oxa-53	Class D β-lactamase	β-lactamase	AS, EW
oxa-5	Class D β-lactamase	β-lactamase	AS, EW
oxa-9	Class D β-lactamase	β-lactamase	AS
oxa-10, oxa-56	Class D β-lactamase	β-lactamase	AS, EW
oxa-12	Class D β-lactamase	β-lactamase	AS, EW
oxa-20	Class D β-lactamase	Amo, Tic	EW
oxa-22	Class D β-lactamase	Benzylpenicillin, Ob	AS, EW
oxa-27	Class D β-lactamase	β-lactamase	AS
oxa-40	Class D β-lactamase	Amo, Tic, Caz, Fep, Cpo, Prl, Kf, Cxm, Ipm	AS, EW
oxa-46, oxa	Class D β-lactamase	Amp, Car, Mez, Kf	AS, EW
oxa-48	Class D β-lactamase	Amo, Tic, Fep, Ipm, Cpo, Prl, Ctx	AS, EW
oxa-50	Class D β-lactamase	Amp, Tic, Ctx, Prl, Kf, Cxm	AS, EW
*oxa-58**	Class D β-lactamase	Amo, Tic, Cpo, Prl, Ipm, Kf	AS, EW
oxa-75	Class D β-lactamase	Amp, Prl	AS, EW

*Gene name**	Gene product	Resistance to	Detected in water environments
cmlA1, cmlA5	Chloramphenicol efflux protein	Cm	AS
cmlB	Hydrophobic polypeptide	Cm	AS
cmxA	Chloramphenicol export protein	Cm	AS, EW
fexA	Florfenicol/chloramphenicol exporter	Cm, Ffc	AS
floR, cmlA	Efflux protein	Cm, Ffc	AS, EW, DW, NW, SW
cat	Chloramphenicol acetyltransferase	Cm	AS, EW
cat	Chloramphenicol acetyltransferase	Cm	AS, EW
cat	Chloramphenicol acetyltransferase	Cm	AS, EW
cat	Chloramphenicol acetyltransferase	Cm	AS, EW
cat2, catII, cmlA	Chloramphenicol acetyltransferase	Cm	AS, EW, SW
catIII	Chloramphenicol acetyltransferase	Cm	AS, EW, NW
catA	Chloramphenicol acetyltransferase	Cm	AS, EW
catB2	Chloramphenicol acetyltransferase	Cm	AS, EW
catB4	Chloramphenicol acetyltransferase	Cm	AS, EW
catB6	Chloramphenicol acetyltransferase	Cm	EW
catB7	Chloramphenicol acetyltransferase	Cm	AS, EW
catB8	Chloramphenicol acetyltransferase	Cm	AS, EW
cat-TC, cat	Chloramphenicol acetyltransferase	Cm	AS, EW
qnrA3, qnr*	Pentapeptide family, DNA-gyrase and topoisomerase IV protection	Nal	AS, EW
qnrB1, qnrB2, qnrB5*	Pentapeptide family, DNA-gyrase and topoisomerase IV protection	Cip	AS, EW
qnrB4	Pentapeptide family	Quinolones	AS, EW
*qnr, qnrS2**	Quinolone resistance determinant	Cip, Nor, Nal	AS, EW
ereA2, ereA	Erythromycin esterase type I	Em	AS, EW
ereB	Erythromycin esterase type II	Em	AS
mph(B)	Macrolide phosphotransferase	Azi, Cla, Em, Rox, Tyl	AS, EW
mph(A)	Macrolide 2'-phosphotransferase I	Azi, Cla, Em, Rox	AS, EW
mph	Macrolide 2'-phosphostransferase	Em	AS, EW
mph(B)	Macrolide 2'-phosphotransferase II	Macrolides	AS, EW
mphBM	Macrolide 2'-phosphotransferase II	Macrolides	AS
ermA	rRNA adenine N6-methyltransferase	Em	EW, SW
ermB	rRNA adenine N6-methyltransferase	Em	EW, SW
ermF	rRNA adenine N6-methyltransferase	MLS	EW, SW
erm (C, E, T, V, X)	rRNA adenine N6-methyltransferase	MLS	EW, SW
mef(A)	Macrolide-efflux protein, MFS permease	Em	AS
mefE, mefI	Macrolide-efflux protein, MFS permease	Em	AS
mel	Macrolide-efflux protein, macrolide-specific ABC-type efflux carrier	Azi, Cla, Em	AS, EW
msrA	Erythromycin resistance ATP-binding protein MsrA	Em	AS

*Gene name**	Gene product	Resistance to	Detected in water environments
arr2	Putative rifampicin ADP-ribosyltransferase	Rif	AS, EW
sulA	Dihydropteroate synthetase	Sul	SD
sulI	Dihydropteroate synthetase	Sul	AS, EW, DW, NW, SD, SW
sulII	Dihydropteroate synthetase	Sul	AS, EW, DW, NW, SD, SW
sul3	Dihydropteroate synthetase	Sul	AS, EW, NW, SD
dfrII	Dihydrofolate reductase	Tp	AS, EW
dfrV	Dihydrofolate reductase	Tp	AS, EW
dfr5, dfr7	Dihydrofolate reductase	Tp	NW
dfr12	Dihydrofolate reductase	Tp	DW, NW, SW
dfr13(dfrXIII)	Dihydrofolate reductase	Tp	AS, EW
dfr15	Dihydrofolate reductase	Tp	EW, NW
dfr16	Dihydrofolate reductase	Tp	AS, EW
dfr17, dfrVII	Dihydrofolate reductase	Tp	DW, NW
dfr18	Dihydrofolate reductase	Tp	NW
dfrA19	Dihydrofolate reductase	Tp	AS, EW
dfrB2	Dihydrofolate reductase	Tp	AS, EW
dfrD	Dihydrofolate reductase	Tp	AS, EW
dhfr1	Dihydrofolate reductase	Tp	AS, EW
dhfrVIII	Dihydrofolate reductase	Tp	AS, EW
dhfrXV	Dihydrofolate reductase	Tp	AS, EW
tetA	MFS tetracycline efflux	Tc	AS, EW, DW, EW, NW, SD, SW, US
tetA	MFS tetracycline efflux	Tc	AS, EW
tetA	MFS tetracycline efflux	Tc	AS, EW
tetA	MFS tetracycline efflux	Tc	AS, EW
tetA (41)	MFS tetracycline efflux	Tc	NW
tetB	MFS tetracycline efflux	Tc	AS, DW, EW, NW, SW, US
tetC	MFS tetracycline efflux	Tc	AS, EW, SW, US
tetD	MFS tetracycline efflux	Tc	AS, EW, DW, EW, SW, US
tetE	MFS tetracycline efflux	Tc	AS, EW, SD, SW, US
tetG	MFS tetracycline efflux	Tc	AS, EW, SW, US
tetH	MFS tetracycline efflux	Tc	AS, EW, SW
tetJ	MFS tetracycline efflux	Tc	SW
tetL	MFS tetracycline efflux	Tc	AS
tet(U)	Replication	Low level Tc	AS, EW
tetY	MFS tetracycline efflux	Tc	AS, EW, SW
tetZ	MFS tetracycline efflux	Tc	SW
tet33	MFS tetracycline efflux	Tc	SW
otrB	MFS tetracycline efflux	Tc	AS, NW, SW
tetR(31)	Tetracycline repressor protein	Regulates expression of	AS, EW

*Gene name**	Gene product	Resistance to	Detected in water environments
		TetA(31)	
effJ (tet(35))	Putative tetracycline efflux pump	Tc	EW
tet(39)	MFS tetracycline efflux	Tc	AS, EW, SD, SW
tetB(P)	GTP-binding elongation factor protein, TetM/TetO family	Tc	AS, SD, SW
tet(M)	GTP-binding elongation factor protein, TetM/TetO family	Tc	AS, EW, NW, SD, SW, US
tet(M)	GTP-binding elongation factor protein, TetM/TetO family	Tc	AS, EW
tet(M)	GTP-binding elongation factor protein, TetM/TetO family	Tc	AS, EW
tet(M)	GTP-binding elongation factor protein, TetM/TetO family	Tc	AS, EW
tet(O)	GTP-binding elongation factor protein, TetM/TetO family	Tc	AS, EW, NW, SD, SW, US
tet(Q)	GTP-binding elongation factor protein, TetM/TetO family	Tc	AS, EW, NW, SW, US
tet(S)	GTP-binding elongation factor protein, TetM/TetO family	Tc	AS, EW, NW, SW, US
tet(T)	GTP-binding elongation factor protein, TetM/TetO family	Tc	SD, SW
tet(W)	GTP-binding elongation factor protein, TetM/TetO family	Tc	SD, NW, SW
otrA	GTP-binding elongation factor protein, TetM/TetO family	Tc	AS, NW, SW
tet(32)	GTP-binding elongation factor protein, TetM/TetO family	Tc	AS
tet(36)	Ribosomal protection tetracycline resistance protein	Tc	EW
tet(X)	Inactivation of tetracycline	Tc	AS, EW
qacB	Permease of the MFS family, multidrug efflux protein	Multidrug efflux	AS
qacED1	Small multidrug resistance protein, membrane transporter of cations and cationic drugs	QAC	AS, EW
qacF	Small multidrug resistance protein, membrane transporter of cations and cationic drugs	QAC	AS
qacF, qacH	Small multidrug resistance protein, membrane transporter of cations and cationic drugs	QAC	AS, EW
qacG2	Small multidrug resistance protein, membrane transporter of cations and cationic drugs	QAC	AS, EW
acrB	RND family, acridine/multidrug efflux pump	Multidrug efflux	AS, EW
acrD	Cation/multidrug efflux pump	Aminoglycosides, Nv	AS, EW
mexB	Cation/multidrug efflux pump, RND multidrug efflux transporter	Multidrug efflux	AS, EW
mexD	RND multidrug efflux transporter	Em, Rox	AS, EW
mexD	Cation/multidrug efflux pump, RND multidrug efflux transporter	Multidrug efflux	AS, EW
mexF	Cation/multidrug efflux pump, RND multidrug efflux transporter	Multidrug efflux	AS, EW
mexI	Cation/multidrug efflux pump, RND	Multidrug efflux	AS, EW

*Gene name**	Gene product	Resistance to	Detected in water environments
	multidrug efflux transporter		
mexY	Cation/multidrug efflux pump, RND multidrug efflux transporter	Multidrug efflux	AS, EW
orf11	ABC type permease	Nal, Nor	AS, EW
kikA	Killing in Klebsiella	IncN-specific gene	AS, EW
oriV	Origin of vegetative replication	IncW-specific region	EW
oriV	Origin of vegetative replication	IncQ-specific region	AS, EW
rep	Replication initiation protein	IncA/C-specific gene	AS, EW
repE	Replication initiation protein	IncFIA-specific replication gene	AS, EW
trfA	Replication initiation protein	Initiation of replication, IncP-specific gene	AS, EW

[a] Summarized from the references (Szczepanowski et al., 2009; Zhang et al., 2009)
*Resistance genes recently described in clinical isolates.
Abbreviations: Ak, amikacin; Amo, amoxicillin; Amp, ampicillin; Atm, aztreonam; Azm, azithromycin; Car, carbenicillin; Caz, ceftazidim; Cec, cefaclor; Cfp, cefoperazon; Cft, ceftibuten; Cip, ciprofloxacin; Clr, clarithromycin; Cm, chloramphenicol; Cpo, cefpirom; Cro, ceftriaxon; Ctx, cefotaxime; Cxm, cefuroxime; Em, erythromycin; Fep, cefepim; Ffc, florfenicol; Fox, cefoxitin; Gm, gentamicin; Ipm, imipenem; Kf, cephalothin; Km, kanamycin; Lev, levofloxacin; Met, meticillin; MLS, macrolide-lincosamide-streptogramin B; Mez, mezlocillin; Nal, nalidixic acid; Neo, neomycin; Nor, norfloxacin; Nv, novobiocin; Ob, cloxacillin; Ofx, ofloxacin; Pen-G, penicillin G; Prl, piperacillin; QAC, quaternary ammonium compounds; Rif, rifampicin; Rox, roxithromycin; Spar, sparfloxacin; Sm, streptomycin; Sp, spectinomycin ; Sul, sulfonamides; Tc, tetracyclines; Tic, ticarcillin; Tob, tobramycin; Tp, trimethoprim; Ty, tylosin.
The antibiotic resistance genes were detected in the following water environments: SW special wastewater from hospital, animal production, and aquaculture area; US untreated sewage; AS activated sludge of sewage treatment plant; EW effluent water of sewage treatment plant; NW natural water; SD sediments; and DW drinking water

2.5 Concluding remarks

Due to the massive production and improper usage of antibiotics since 1940s, large numbers of antibiotic resistance genes have been developed and distributed in the biosphere microorganisms, which has overwhelmed the therapeutic usage of antibiotics used today. However, limited knowledge on the roles of antibiotics and antibiotic resistance in nature has hampered the efforts to prevent and control the antibiotic resistance. It is recently updated that the sub-inhibitory concentrations of antibiotics may play a universal signaling role to select adaptive phenotypic and genotypic microbes in various ecosystems. Nevertheless, the causal relationship between the low concentrations of antibiotics detected in the environment and the proliferation of antibiotic resistance genes is not identified yet, neither the effects of the sub-inhibitory concentrations of antibiotics on the ecological function disturbance and on the selection of resistant microorganisms in the environment. Therefore, it would be essential to investigate the impacts of low concentrations of antibiotics (e.g. erythromycin) on the micro-ecology of WWTPs, which are the main reservoirs of the antibiotics discharged by anthropogenic activities.

Chapter 3

Influence of Trace ERY and ERY-H_2O on Carbon and Nutrient Removal and on Resistance Selection in SBRs

3.1 Abstract

Three SBRs were operated in parallel to study the effects of trace ERY and ERY-H_2O on the treatment of a synthetic wastewater. Through monitoring (1) daily effluents and (2) concentrations of N and P in certain batch cycles of the three reactors operated from transient to steady states, the removal of carbon, N, and P was affected negligibly by ERY (100 µg/L) or ERY-H_2O (50 µg/L) when compared with the control reactor. However, through analyzing microbial communities of the three steady state SBRs on high-density microarrays (PhyloChip), ERY, and ERY-H_2O had pronounced effects on the community composition of bacteria related to N and P removal, leading to diversity loss and abundance change. The above observations indicated that resistant bacteria were selected upon exposure to ERY or ERY-H_2O. Short-term batch experiments further proved the resistance and demonstrated that ammonium oxidation (56–95%) was inhibited more significantly than nitrite oxidation (18–61%) in the presence of ERY (100, 400, or 800 µg/L). Therefore, the presence of ERY or ERY-H_2O (at µg/L levels) shifted the microbial community and selected resistant bacteria, which may counteract the influence of the antibiotic ERY or its derivative ERY-H_2O (at µg/L levels) on carbon, N, and P removal in the SBRs.

3.2 Introduction

Macrolide antibiotics are among the most important and common antibacterial agents applied to both human and animal diseases caused by bacteria (Dubois et al., 2001; Pothuluri et al., 1998). Macrolides prevent bacterial growth by binding to the 23S ribosomal RNA (rRNA) of the large (50S) ribosomal subunit so as to inhibit protein synthesis in the elongation step (Verdier et al., 2001). Though macrolides can be metabolized by demethylation in the body, the urinary excretion of unchanged

parent drugs could reach 5–10% for ERY, 10–20% for clarithromycin, 30% for roxithromycin, 10–20% for spiramycin, and 6–12% for azithromycin (Forth et al., 1992). Unlike other macrolide members, ERY can be inactivated easily by gastric acid to form ERY-H_2O by losing one molecule of water (McArdell et al., 2003). Although ERY-H_2O has negligible antibacterial activity, it may be involved in inducing bacterial resistance to the parent drug (Majer, 1981). The excreted urine, carrying the active antibiotics and their metabolites, will be finally discharged to the wastewater treatment systems.

In contrast to the macrolide antibiotics working principle, mechanisms of bacterial resistance can be the excretion of antibiotic drugs by efflux pumps or the alteration of the target site to avoid binding of these drugs (Amin et al., 2006). To gain resistance, bacteria can also destruct or modify the macrolides directly by the enzymatic hydrolysis (macrolide esterase) and group transfer strategies (phosphate transfer and glycosyltransfer) (Wright, 2005). Therefore, the massive use of the macrolide antibiotics has raised questions on the possible long-term consequences of resistance selection because trace levels of macrolides have been detected in aquatic environments (Giger et al., 2003; Hirsch et al., 1999; Karthikeyan and Meyer, 2006; Kolpin et al., 2002; McArdell et al., 2003; Richardson et al., 2005; Xu et al., 2007b). As one of the macrolide antibiotics, ERY was measured as ERY-H_2O in most studies because ERY could be transformed to ERY-H_2O in the slightly acidic aquatic environment or during the solid phase extraction at acidic conditions. Dehydrated erythromycin has been detected in most of water or wastewater samples surveyed worldwide, such as America (Karthikeyan and Meyer, 2006; Kolpin et al., 2002), Canada (Miao et al., 2004), Germany (Hirsch et al., 1999), Switzerland (Giger et al.,

2003; McArdell et al., 2003), Taiwan (Lin and Tsai, 2009), Hong Kong (Gulkowska et al., 2008), and China (Richardson et al., 2005; Xu et al., 2007b).

It was speculated that the ERY-resistant pathogenic bacteria could be developed even at low ERY concentrations (less than 2 μg/L) in WWTPs, which breed a much wider range of environmental bacteria than the natural aquatic environment (Hirsch et al., 1999). Although the potential effects of ERY, at ultra-low concentrations (μg/L), on WWTPs have attracted the attention from the public and scientific communities, few investigations have been made so far. Most studies have been conducted on the detection of ERY-H_2O at low concentrations in the WWTPs (Giger et al., 2003; Hirsch et al., 1999; Karthikeyan and Meyer, 2006; McArdell et al., 2003). Usually, the resistance or susceptibility of cultures to ERY is judged by inhibitory concentrations. For example, it is reported that the inhibitory concentrations of ERY to selected pure cultures were 0.01–10 mg/L and 0.1–>200 mg/L to the Gram-positive and Gram-negative organisms, respectively (Oleinick, 1975). Nimenya (1999) concluded the highly significant linear correlation between dose of ERY (10, 20, 40, and 80 mg/L) and its inhibition on ammonia removal (correlation coefficient r=0.890, probability value p<0.05) and nitrate production (r=0.937, p<0.05), in which the reported inhibition percentage of ammonium oxidation and nitrate production was 0.20% and 0.28% at ERY of 1 mg/L. Additionally, the influence of ERY at relatively high concentrations in the pharmaceutical wastewater was studied in anaerobic reactors. For example, Amin (2006) suggested that the development of antibiotic resistance alleviated the inhibition of ERY (1–200 mg/L) on biogas production and on butyric acid conversion in an anaerobic lab scale reactor with complex microbial communities, accompanying with concentrations of ERY in the effluent ranging from 0.4–150 mg/L. In order to reach

the discharge standard of chemical oxygen demand (COD), effluent of anaerobic processes is always treated by aerobic processes, in which ERY might be further removed.

The aim of this study was to assess the influence of ERY or ERY-H_2O at low concentrations (μg/L) on the carbon, nitrogen, and phosphorous removal in SBRs. The inhibitory effects on carbon and nutrients removal were evaluated by a long-term operation of three SBRs, namely R1, R2, and R3 fed with the same synthetic wastewater but spiked with 50 μg/L of ERY-H_2O, 100 μg/L of ERY and no antibiotics, respectively. The microbial communities of the three steady state SBRs were analyzed by employing high-density phylogenic 16S rRNA gene microarrays (PhyloChip) containing 1,440 distinguishable prokaryotic operational taxonomic units (OTUs), and the community shifts in R1 (ERY-H_2O) and R2 (ERY) were compared with that in R3 (control). To verify whether the PhyloChip-observed nitrifying bacteria shifts were correlated with their resistance to ERY, short-term running batch experiments were conducted to study higher concentrations (100, 400, and 800 μg/L) of ERY's inhibition on nitrifying bacteria present in the biomass of the three steady state SBRs.

3.3 Materials and methods

3.3.1 Startup and operation of SBRs

Three SBRs (4 L) were started up with the same seeding sludge from a mother reactor (MR). The MR was seeded with sludge collected from an aeration tank of a local domestic WWTP. Before being inoculated to the three SBRs, the sludge in MR was fed with synthetic wastewater for 8 months to minimize the residue inhibitory and resistant effects of antibiotics possibly existing in the local domestic wastewater, as well as, to achieve optimal nitrogen and phosphorus removal by adjusting

operation parameters, such as solids retention time (SRT), hydraulic retention time (HRT), aeration, and batch modes.

The synthetic wastewater consisted of total organic carbon (TOC) of 225 mg/L (equal to theoretical COD of 600 mg/L) from glucose of 563 mg/L, NH_4^+–N of 60 mg/L from NH_4Cl of 229 mg/L, PO_4^{3-}–P of 15 mg/L from K_2HPO_4 of 84 mg/L, alkalinity of 420 mg/L from $NaHCO_3$ of 706 mg/L, and the trace elements in mg/L (EDTA 3; $Na_2MoO_4 \cdot 2H_2O$ 1.23; $CaSO_4 \cdot 2H_2O$ 24.6; $MgSO_4$ 6; $CoCl_2 \cdot 6H_2O$ 4; $FeCl_3 \cdot H_2O$ 15; $CuSO_4 \cdot 5H_2O$ 0.002; $MnSO_4 \cdot H_2O$ 0.16; $ZnSO_4 \cdot 7H_2O$ 0.002; KI 0.002; and H_3BO_3 0.002). The MR was fed with synthetic wastewater in half-concentration all the time, while the three SBRs were fed with the synthetic wastewater in half-concentration only during the first 34 days to model the wastewater at relative low concentrations, and then increased to full concentration from day 35 to represent the wastewater at relative high concentrations. During the entire operation, the SBRs were spiked with ERY-H_2O of 50 µg/L (R1), ERY of 100 µg/L (R2), and no antibiotics (R3), respectively. The concentrations of ERY and ERY-H_2O in this study are all in the µg/L range, which can be found in the effluent of plants treating pharmaceutical wastewater or in the hospital effluents. The reason to choose relatively higher concentration of ERY (100µg/L) than ERY-H_2O (50µg/L) is that ERY could be degraded to ERY-H_2O at slightly acidic conditions. By adding a little more ERY, the microbes in the reactors are always exposed to it though some degradation may occur under unpleasant conditions. From day 56 to 62, the wasted sludge collected from R2 (ERY) was used to set up a fourth reactor R2'. The R2' (fed with ERY of 100 µg/L) was operated as the same as R2 except that it was applied with a shock loading of high TOC up to 6,000 mg/L on day 66, which commonly encountered in pharmaceutical wastewater treatment plants (Amin et al., 2006; Yang et al., 2009),

following by various TOC loading until day 130. The TOC and nitrogen contributed by ERY-H_2O or ERY were negligible (about 0.6 g TOC and 0.02 g N/g ERY or ERY-H_2O). Erythromycin (potency≥850 μg/mg) and all other chemicals were supplied by Sigma-Aldrich (Singapore). Erythromycin stock solution was prepared in 2mM $NaHCO_3$ (pH=7.3) because its degradation rate increases logarithmically with a decrease of pH from 7.3 to 3.5 (Kim et al., 2004). Dehydrated erythromycin was prepared according to the previously reported method (Abuin et al., 2006; McArdell et al., 2003).

With an accurate programmable logic controller, the three SBRs were operated simultaneously in the same batch mode of an 8-hour cycle, consisting of inlet (0–14 min), pre-mixing for anoxic/anaerobic condition (0–60 min), aeration for oxic condition (60–300 min), post-mixing for anoxic condition (300–364 min), waste sludge (360–364 min) by decanting ~1/20 volume of 4 L mixed liquid (SRT 6.7 days, which is derived by 20/3 and 3 means three cycles per day), settling (364–404min), decanting supernatant to 2 L (404–440min; HRT 16 hr) and idle (440–480min) (Fig. 3.1). The dilution factor was 2 for the three SBRs by discharging 2 L of liquid from the 4 L reactor in each cycle. The pH ranged from 6.6 to 8.2 during one cycle without adjustment. The stable oxygen supply during aeration guaranteed ~ 5 mg/L of dissolved oxygen (DO) at the end of aeration phase except for R2' (ERY) during the shock load period. All SBRs were operated under ambient temperature (25–27°C).

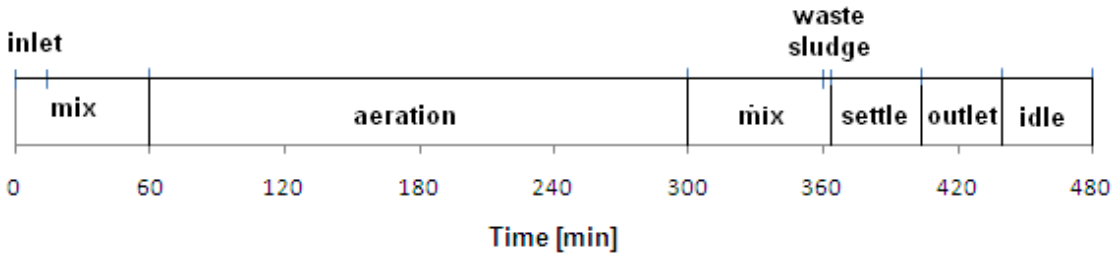


Fig. 3.1 Batch mode of sequencing batch reactors (SBRs).

3.3.2 Batch experiments

To test the long-term resistance selection of nitrifying bacteria exposed to ERY-H_2O (50 µg/L) in R1 and ERY (100 µg/L) in R2, short-term batch experiments of ammonium oxidation and nitrite oxidation were performed in 250 ml flask bottles with a shaking speed of 150 rpm. The ammonium oxidation batches were conducted in thirty-six flasks, which were filled with 50 ml of diluted ATCC medium 2,265 (*Nitrosomonas europaea* medium) to a final concentration of 35 mg/L NH_4^+–N except replacing Na_2CO_3 with $NaHCO_3$ of 490 mg/L. The thirty-six flasks were divided into three groups for inocula from R1 (ERY-H_2O), R2 (ERY), and R3 (control), respectively. Then each group of twelve flasks was spiked with ERY at concentrations of 0 (control), 100, 400, and 800 µg/L in triplicates. The nitrite oxidation batches were conducted in the same way as the ammonium oxidation batches in another thirty-six flasks, but filled with 50 ml of diluted ATCC medium 480 (*Nitrobacter* medium 203) to a final concentration of 21 mg/L NO_2^-–N. Similarly to the above experiments on ERY, ammonium oxidation and nitrite oxidation were conducted on inocula from R1, R2, and R3 in a total of seventy-two flasks spiked with ERY-H_2O at concentrations of 0 (control), 100, 400, and 800 µg/L. The inocula, 1ml of biomass taken from the steady state SBRs, were washed three times with the aforementioned media by centrifugation to minimize previously fed antibiotics and organic carbon, and then were added to the liquid medium (2% by volume of inocula). All inocula were freshly applied in only one batch of experiments to avoid artificially selecting for primarily resistant organisms. The flask bottles were placed on a shaker for mixing and aeration at room temperature (25–27°C).

Statistical analysis of the biomass-source dependent ERY inhibition was carried out by the simple paired Student's *t* test to compare the daily product

concentrations between any two of the three sources biomass during the 3-day period of the batch experiments. Differences were considered significant if the probability values $p \leq 0.05$.

3.3.3 Collection and preparation of samples

During the operation of the three SBRs, equal volumes (5 ml) of effluents from three cycles of each day were collected, and then mixed together as the daily effluent for analysis. Samples in SBRs within one cycle were collected according to online DO and pH signals, which were the indicators for carbon and nitrogen removal (Yang et al., 2007). The frequency of cycle analysis was once for every 3 to 5 days in the transient and the early steady states and decreased to every 7 to 30 days in the later steady states. Samples from SBRs were filtered through a sterile filter membrane (GN-6 Metricel S-Pack, 0.45 μm, 47mm, mixed cellulose ester, Pall Cop., USA) for TOC, nitrification, and phosphate removal analysis. Therefore, the measured TOC, phosphate, and all kinds of nitrogen were in dissolved forms throughout this study. To avoid sorption on membrane during filtration, the samples for antibiotics analysis were centrifuged at 14,000 rpm for 15 min at 25°C. Samples from batch conical flasks were collected daily and centrifuged as above.

3.3.4 Analytical methods

The concentrations of dissolved TOC and dissolved TN were detected on a TOC analyzer with a TN measuring unit (Shimadzu TOC-Vcsh combined with TNM-1 Unit, Japan). Measurement of the dissolved phosphorus (PO_4^{3-}–P) and nitrogen in forms of NO_3^-–N, NO_2^-–N and NH_4^+–N were performed on an ion chromatography (DIONEX DX500 chromatography system, USA). Erythromycin and ERY-H_2O were

analyzed on a high performance liquid chromatography (Agilent 1100 Series, Agilent Technologies, Germany) with electro-spray tandem mass spectrometry (API 2000^{TM}, Applied Biosystems/MDS Sciex, USA; LC-MS-MS). The previously reported LC method (McArdell et al., 2003) was utilized for analysis of ERY and ERY-H_2O on a reverse phase Xbridge^{TM} Phenyl column (50 ×2.1 mm id, 3.5µm, waters Inc., USA.) protected by a guard column (Xbridge^{TM} Phenyl, 10 ×2.1 mm id, 3.5µm, waters Inc., USA.). The MS-MS analysis was performed in the positive ion mode (McArdell et al., 2003). One precursor ion and one product ion were chosen for MS-MS determination, that is, m/z 734.5 and 158.2 for ERY and m/z 716.5 and 158.2 for ERY-H_2O. The detection limits were 10 µg/L for ERY and 8 µg/L for ERY-H_2O.

3.3.5 DNA extraction, polymerase chain reaction and PhyloChip

Approximately 1 ml of mixed liquid was centrifuged (14,000 g, 5 min) and cell pellets were collected for DNA extraction. The genomic DNA was extracted and purified by using DNeasy Tissue Kit (QIAGEN GmbH, Germany), except with modification of bead beating the cells with glass beads (Diameter 0.1mm, Biospec, USA) for better cell lysis. The high-density phylogenic 16S rRNA gene microarrays (PhyloChip) employed in this study contain 1,440 distinguishable prokaryotic OTUs identified by 35,000 probes, which were designed according to the approach described previously (DeSantis et al., 2003). Each DNA pool extracted from triplicate sludge samples was tested on triplicate PhyloChips. The 16S rRNA gene amplification, PhyloChip processing, scanning, OTU scoring, and normalization were all performed as previously described (Brodie et al., 2006; Flanagan et al., 2007).

3.4 Results

3.4.1 Effects of ERY-H_2O on SBR performance

The sequencing batch reactor R1 (fed with 50 µg/L of ERY-H_2O) and R3 (control reactor) reached their first steady state (e.g., constant carbon and nutrients removal) on day 21 when added with TOC of 110 mg/L, NH_4^+–N of 30 mg/L and PO_4^{3-}–P of 7.5 mg/L. After doubling the concentrations of TOC, NH_4^+–N and PO_4^{3-}–P on day 35, R1 (ERY-H_2O) and R3 (control) once again reached their steady states after day 55. Similar carbon and nitrogen removal profiles were observed in R1 (ERY-H_2O) and R3 (control) during the whole running period of 400 days (Fig. 3.2 showed data of 180 days, and Fig. 3.3 showed data of 400 days), which were consistent with the intensive observation on nitrogen removal profile within individual cycle during the steady states (Fig. 3.4). Concentrations of TOC in effluents of R1 (ERY-H_2O) and R3 (control) were below 10 mg/L in most of the days during the operation period, even with doubled concentrations of influent on day 35. The NH_4^+–N concentrations were undetected in the daily effluents for most of the days, indicating that ammonium oxidation was completed and not affected by the increased dosage of both TOC and NH_4^+–N in the influents on day 35. As the intermediate of nitrification and denitrification, NO_2^-–N was not accumulated in the effluents and appeared to be negligible during the aerobic nitrification process. Because the amount of TN and NO_3^-–N decreased in the post-mixing period (Fig. 3.4), endogenous denitrification occurred. Additionally, ERY-H_2O concentration in the effluent of R1 (ERY-H_2O) was always ~ 15 µg/L. The missed 70% of ERY-H_2O (~ 35 µg/L) could be adsorbed to the sludge or degraded by the biomass.

3.4.2 Effects of ERY on SBR performance

Similarly, when comparing the TOC and NH_4^+–N removal of R2 (100 µg/L of ERY) with that of R3 (control), ERY was not found to have any effect on the performance of the reactor during the 400 days operation (Fig. 3.2 and Fig. 3.3). To further understand whether the antibiotic ERY at µg/L levels could cause the performance of reactor beyond recovery at accidental conditions (such as the commonly occurred shock loading of high TOC in pharmaceutical wastewater treatment plants), a TOC of 6,000 mg/L in the influent was supplied to R2' (a duplicate of R2) for 1 week (from day 65 to 72), while R2 (ERY) was operated at normal TOC concentration of 220 mg/L to avoid reactor failure and to minimize possible microbial community changes due to the TOC shock. The results showed that carbon removal in R2' (ERY) was recovered within 18 days after the shock of high TOC, indicating that ERY did not prevent R2' from recovery, though the nutrients of nitrogen (N) and phosphorous (P) were insufficient (Fig. 3.2b' and Fig. 3.3b). The following describes the recovery process. When the concentration of TOC in the influent was reduced to 400 mg/L (from day 73 to 90), the concentration of TOC in the effluent decreased in four levels (3,500 mg/L, 400 mg/L, 200 mg/L and then lower than 50 mg/L on day 82), indicating that the organic carbon was biodegraded rather than by dilution because of a dilution factor of only 2 for the SBRs. After day 91, with enough aeration and overdosed nutrient, carbon removal in R2' (ERY) was recovered totally as TOC concentrations in the effluent dropped to 15 mg/L and maintained below 10 mg/L in later days.

Moreover, during the periods of the shock loading and the recovery phases of R2' (ERY), the results demonstrated typical profiles of nitrification, from nitrite production to complete nitrification (Fig. 3.2e and Fig. 3.3e). The change occurs

commonly when SBRs start nitrification even without antibiotics, since ammonium oxidizing microorganisms (AOMs; like *Nitrosomonas*) grow significantly faster than nitrite oxidizing bacteria (NOB; like *Nitrospira*) (Watson et al., 1989). When R2' (ERY) was fed with excess amount of ammonium than assimilation required on day 91, the NO_2^-–N and NO_3^-–N appeared in the effluent with the decrease of NH_4^+–N from day 100, indicating that nitrification resumed. The reduction of TN observed from day 100 to day 110 suggests that denitrification resumed from day 100 as well, and reached an equilibrium from day 110 in R2' (ERY). Interestingly, the intermediate NO_2^-–N appeared to be a dominant nitrogen product from day 100 to day 106, which was taken over by NO_3^-–N as usual after day 106. The transit of nitrification processes was clearly confirmed by the accumulation of NO_2^-–N in the aeration period as observed within the cycles on day 119, while not in cycles on day 130 (Fig. 3.4).

Additionally, during the whole operation period, the concentrations of ERY in the effluent of R2 (ERY) were below the detection limit (10 µg/L), and ERY-H_2O which could be produced from ERY was not detected in most of the days either (detection limit of 8 µg/L). Therefore, the missed ERY might be attributed by the sorption to the biomass in R2 (ERY) or degradation to compounds other than ERY-H_2O.

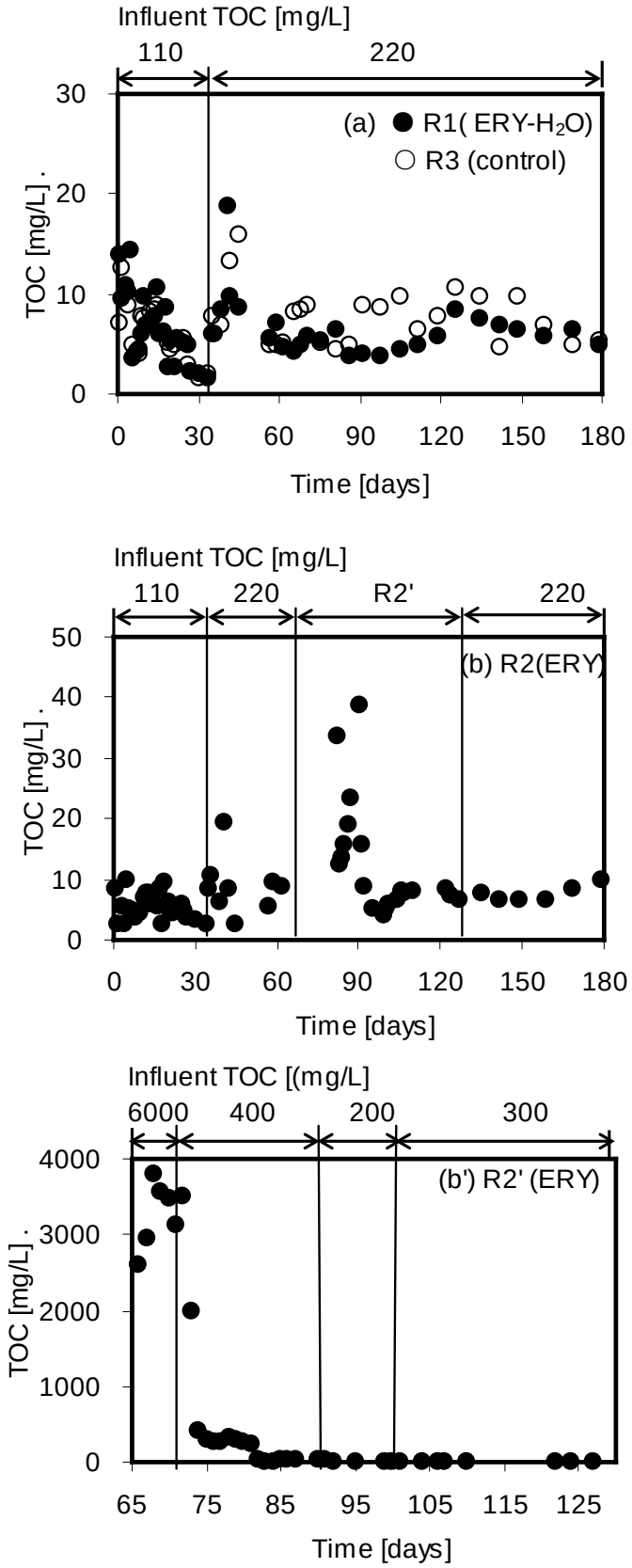
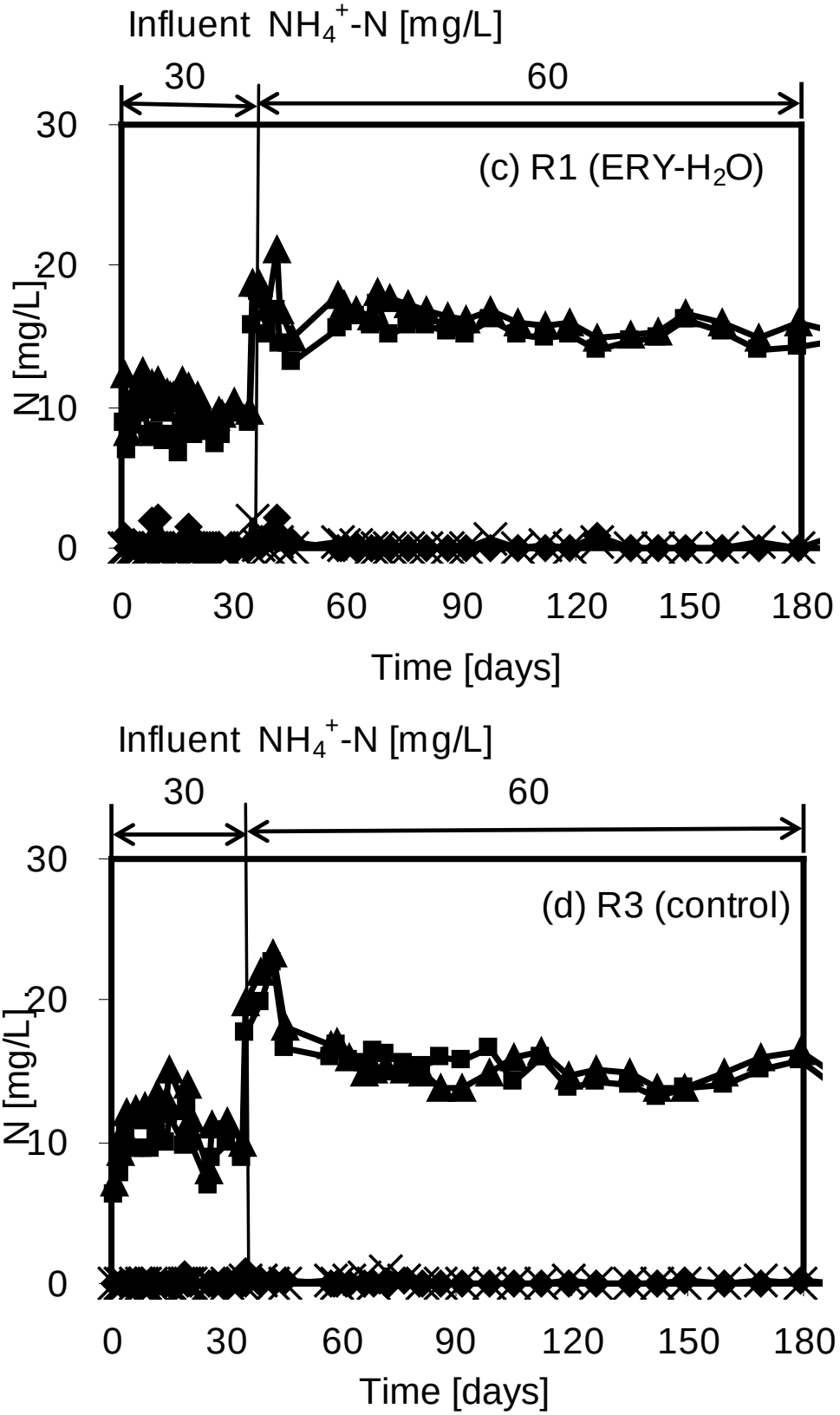
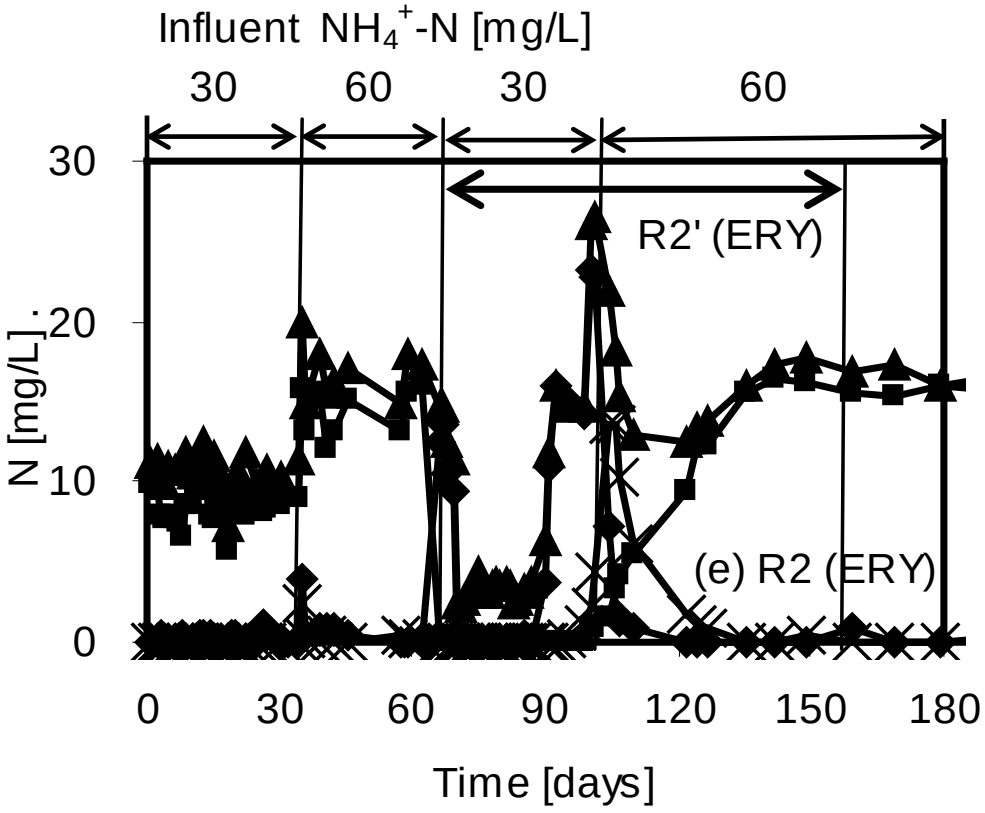


Fig. 3.2 180-day daily effluents of R1 (ERY-H_2O of 50 µg/L), R2 or R2' (ERY of 100 µg/L), and R3 (control): the averages of soluble TOC (●) or (○) in daily effluents consist of equal volumes of effluents from three cycles of each day



Continued Fig. 3.2 180-day daily effluents of R1 (ERY-H_2O of 50 µg/L) and R3 (control): the averages of soluble TN (▲), NO_3^-–N (■), NO_2^-–N (×), and NH_4^+–N (◆) in daily effluents consist of equal volumes of effluents from three cycles of each day



Continued Fig. 3.2 180-day daily effluents of R2 or R2' (ERY of 100 µg/L): the averages of soluble TN (▲), NO_3^-–N (■), NO_2^-–N (×), and NH_4^+–N (◆) in daily effluents consist of equal volumes of effluents from three cycles of each day

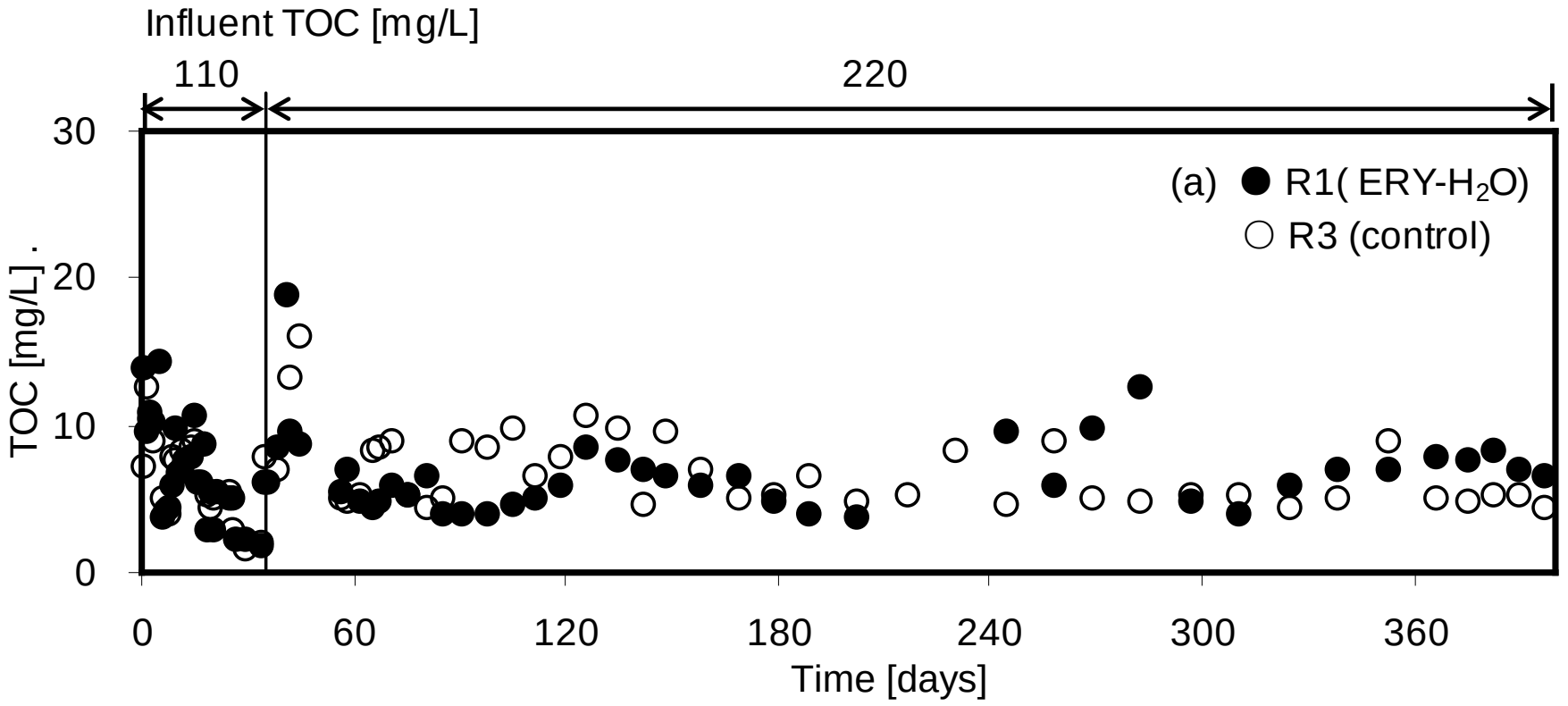
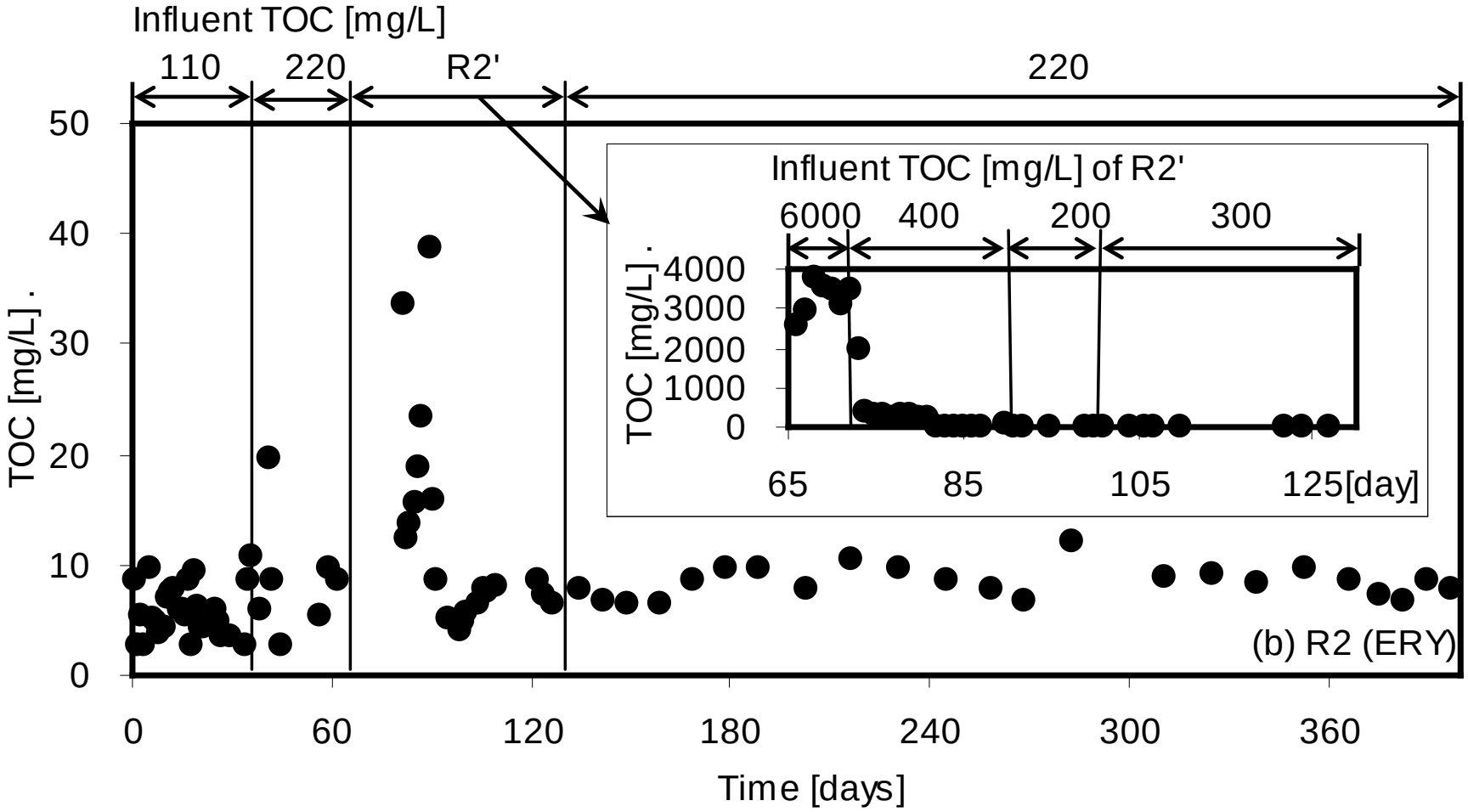
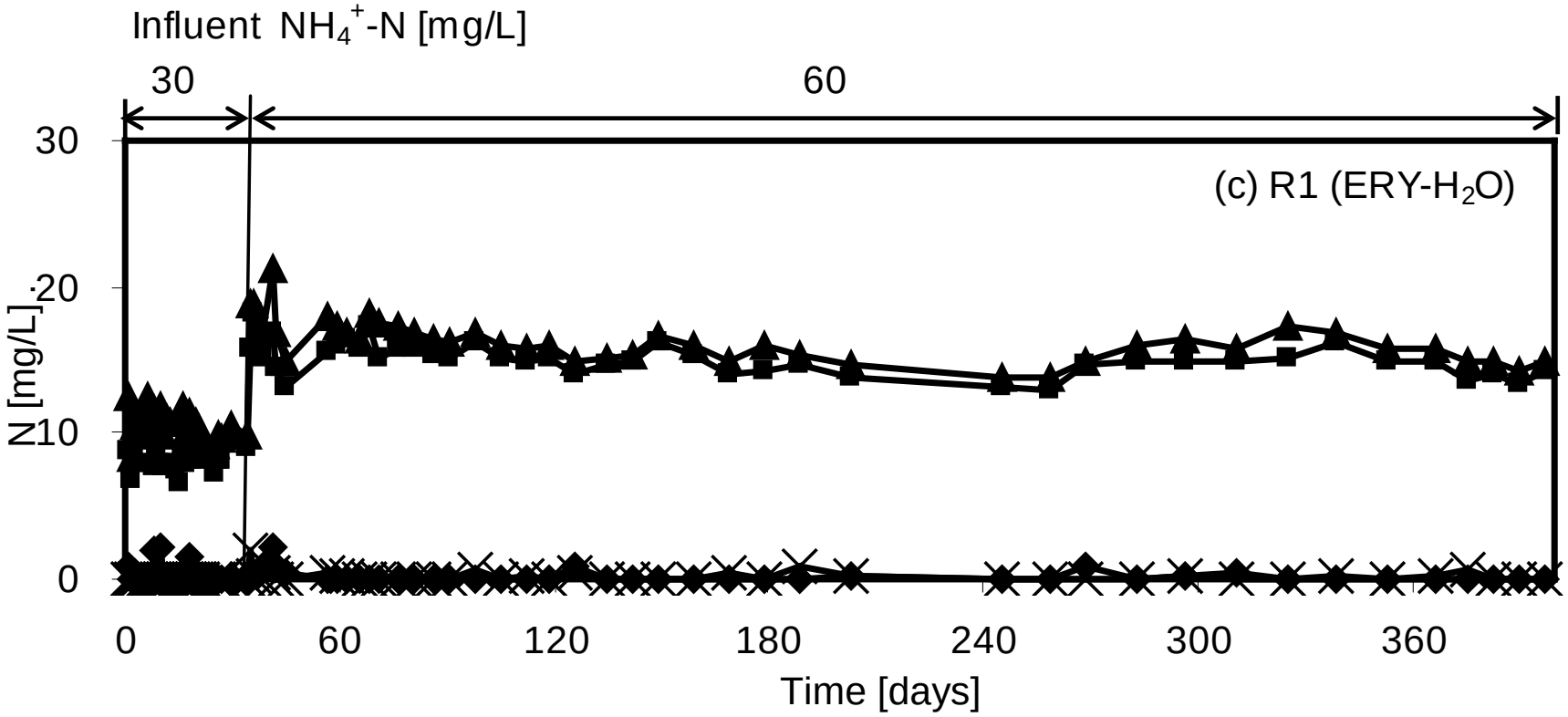


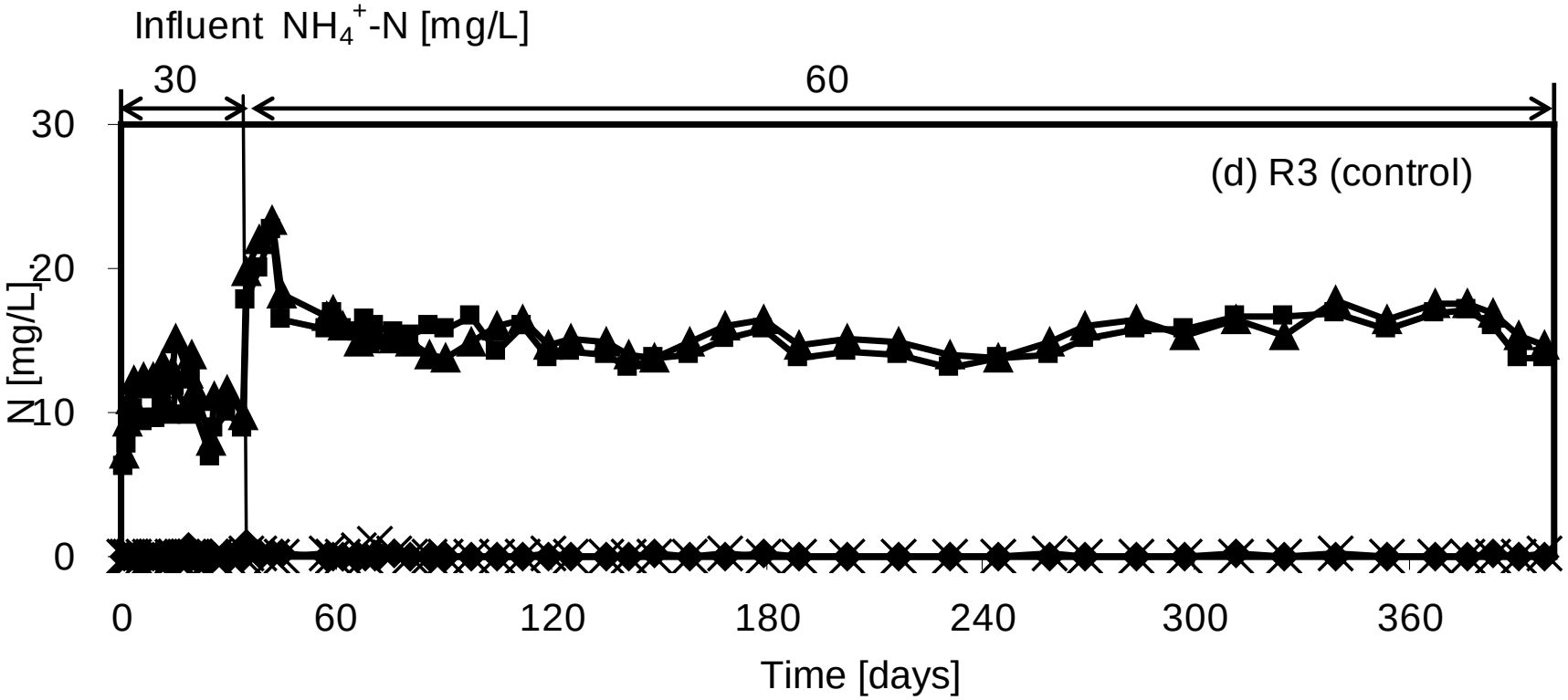
Fig. 3.3 400-day daily effluents of R1 (ERY-H_2O of 50 µg/L) and R3 (control): the averages of soluble TOC (●) or (○) in daily effluents consist of equal volumes of effluents from three cycles of each day



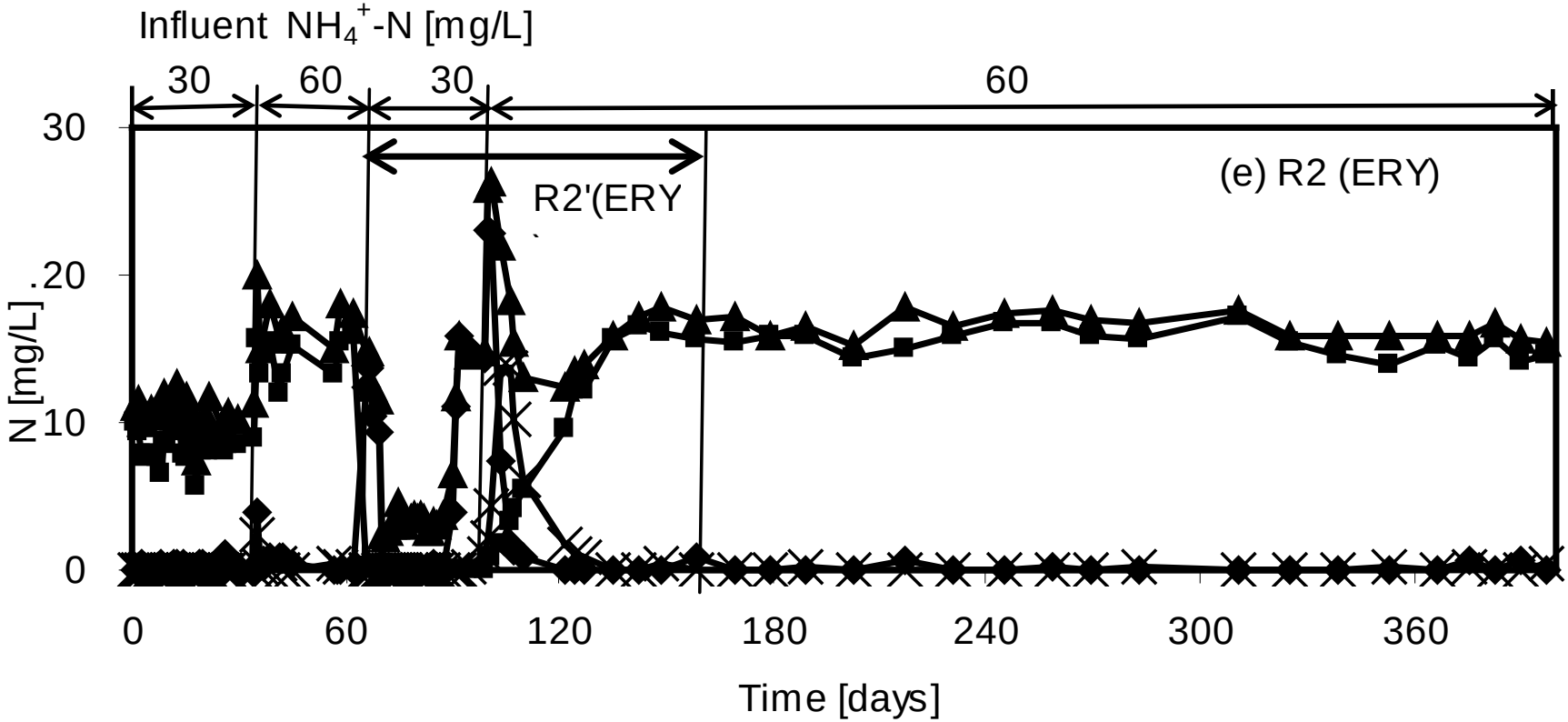
Continued Fig. 3.3 400-day daily effluents of R2 or R2' (ERY of 100 μg/l),: the averages of soluble TOC (●) in daily effluents consist of equal volumes of effluents from three cycles of each day



Continued Fig. 3.3 400-day daily effluents of R1 (ERY-H_2O of 50 µg/L): the averages of soluble TN (▲), NO_3^-–N (■), NO_2^-–N (×), and NH_4^+–N (◆) in daily effluents consist of equal volumes of effluents from three cycles of each day



Continued Fig. 3.3 400-day daily effluents of R3 (control): the averages of soluble TN (▲), NO_3^-–N (■), NO_2^-–N (×), and NH_4^+–N (◆) in daily effluents consist of equal volumes of effluents from three cycles of each day



Continued Fig. 3.3 400-day daily effluents of R2 or R2' (ERY of 100 µg/l): the averages of soluble TN (▲), NO_3^-–N (■), NO_2^-–N (×), and NH_4^+–N (◆) in daily effluents consist of equal volumes of effluents from three cycles of each day

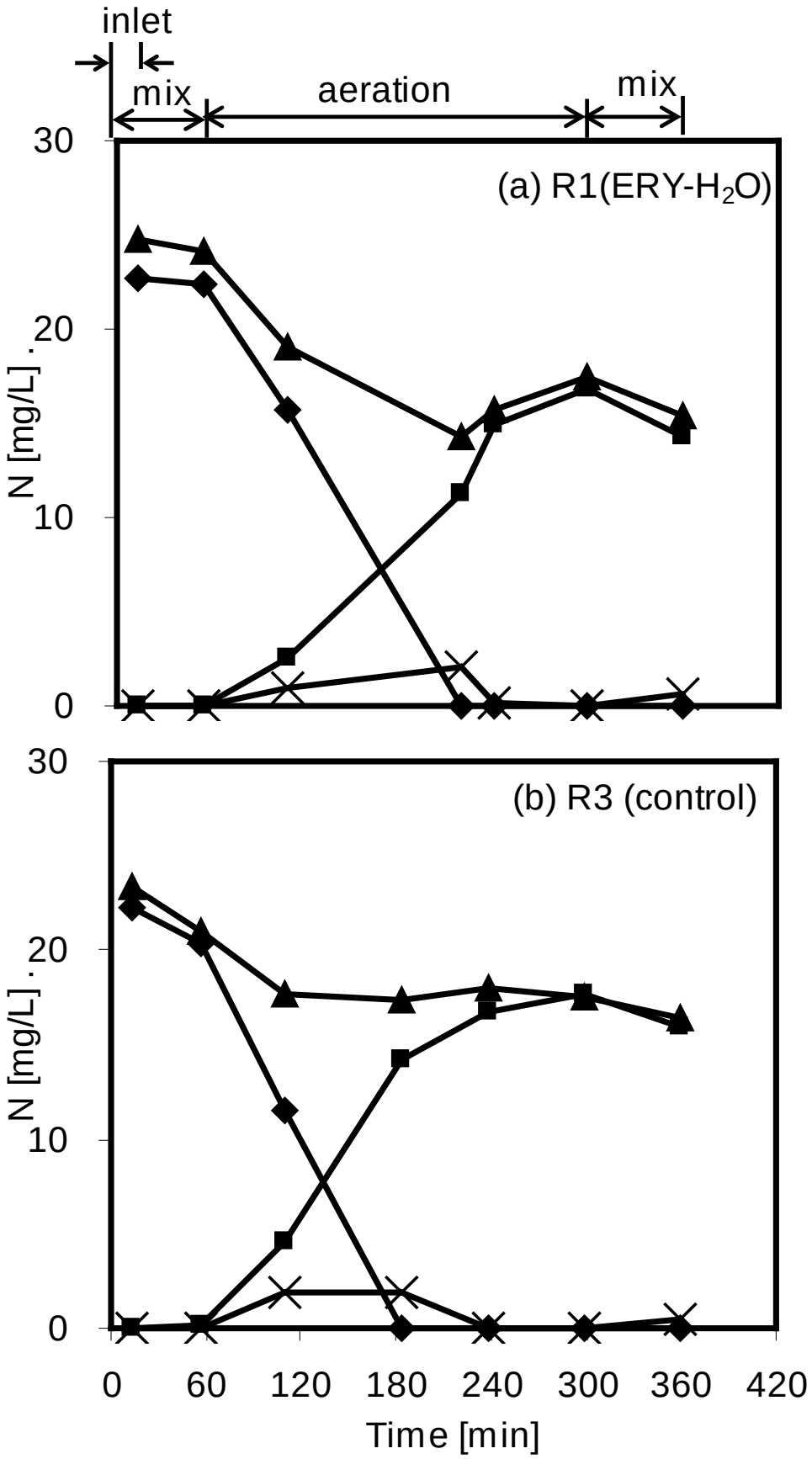
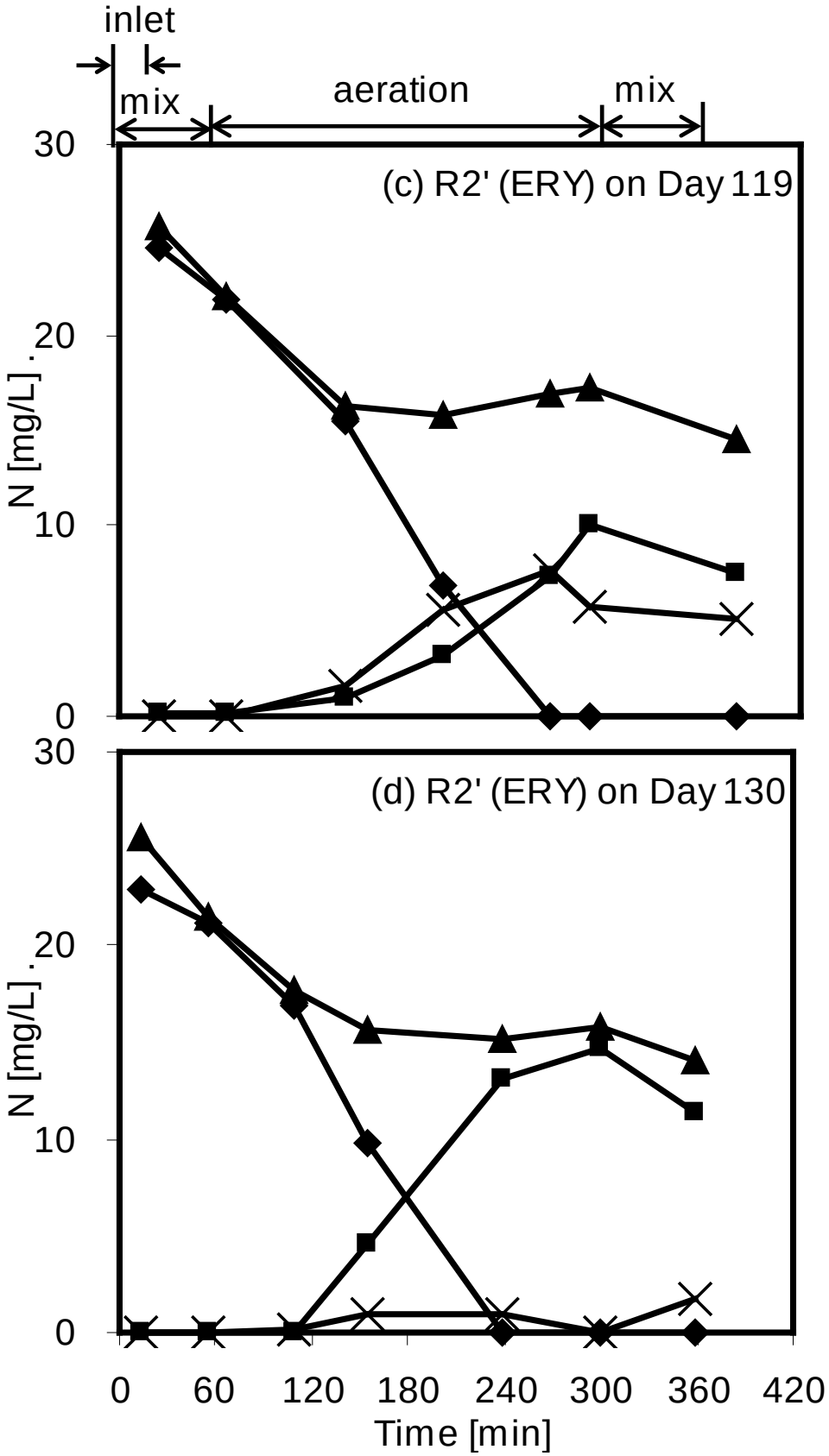


Fig. 3.4 Comparison of nitrogen dynamics within the cycles of **a** R1 (ERY-H_2O) and **b** R3 (control) during the steady states: soluble TN (▲), NO_3^-–N (■), NO_2^-–N (×) and NH_4^+–N (◆)



Continued Fig. 3.4 Comparison of nitrogen dynamics within the cycles of **c** R2' (ERY) on day 119, and **d** R2' (ERY) on day 130: soluble TN (▲), NO_3^-–N (■), NO_2^-–N (×) and NH_4^+–N (◆)

3.4.3 Phosphorus removal affected by ERY and ERY-H_2O

Since phosphorus removal is another key parameter to evaluate the performance of the reactors, average concentrations of PO_4^{3-}–P were monitored in the daily effluents of the three SBRs (Fig. 3.5). The averages of PO_4^{3-}–P during the 400 days operation were 3.7, 4.6, and 4.8 mg/L for R1 (ERY-H_2O), R2 (ERY), and R3 (control), respectively. To capture detailed process dynamics for PO_4^{3-}–P concentrations, 8-hour cycles were investigated for all three SBRs in the steady states. In all the cycles investigated, the PO_4^{3-}–P release in R1 (ERY-H_2O) during the 60-minute of premixing period was relatively more than that in R2 (ERY) and R3 (control) (Fig. 3.5). The subtle lower PO_4^{3-}–P concentrations in effluent of R1 (ERY-H_2O) than those in R2 (ERY) and R3 (control) might be due to the higher PO_4^{3-}–P release in R1 (ERY-H_2O) than those in other two reactors.

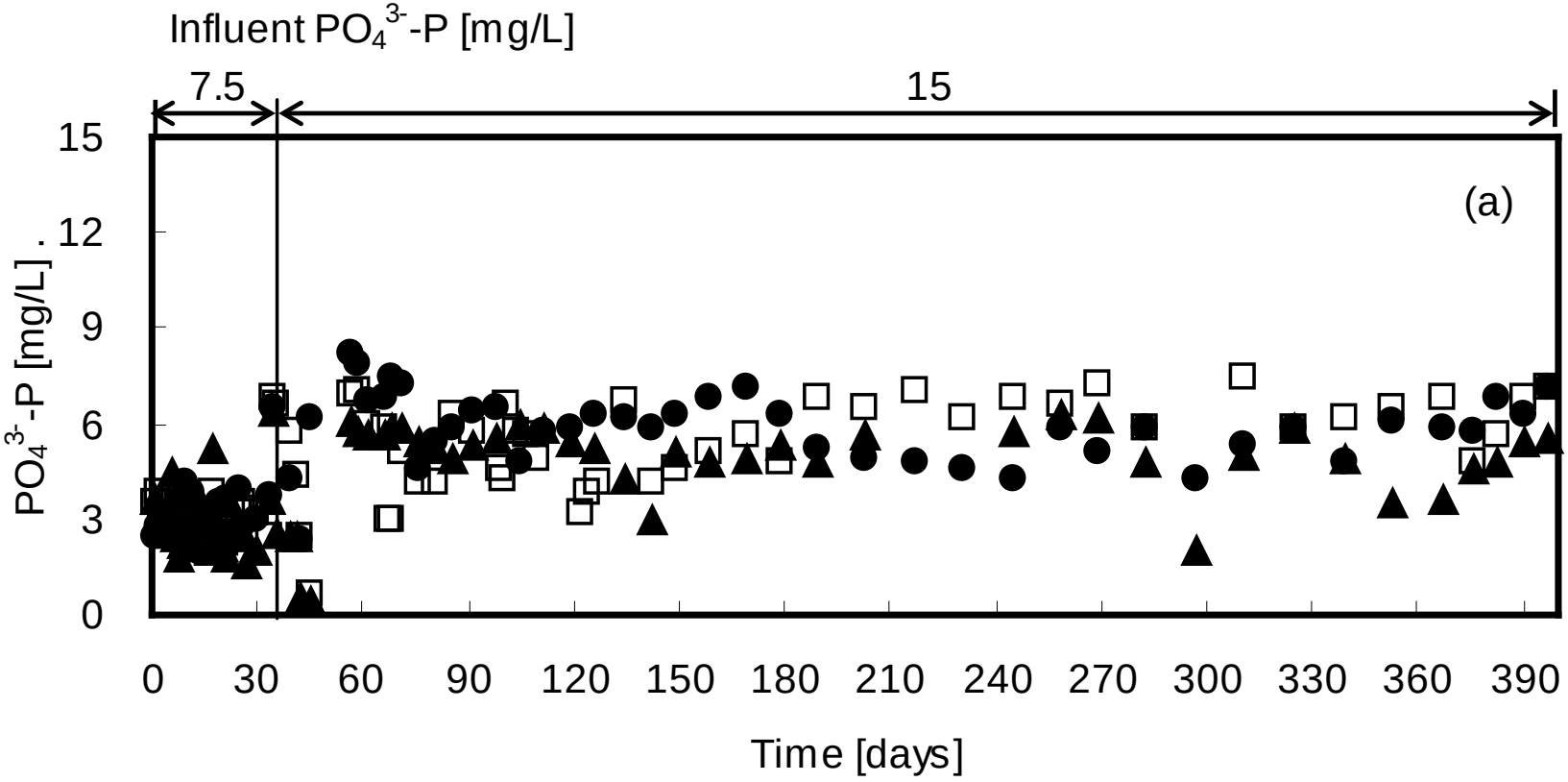
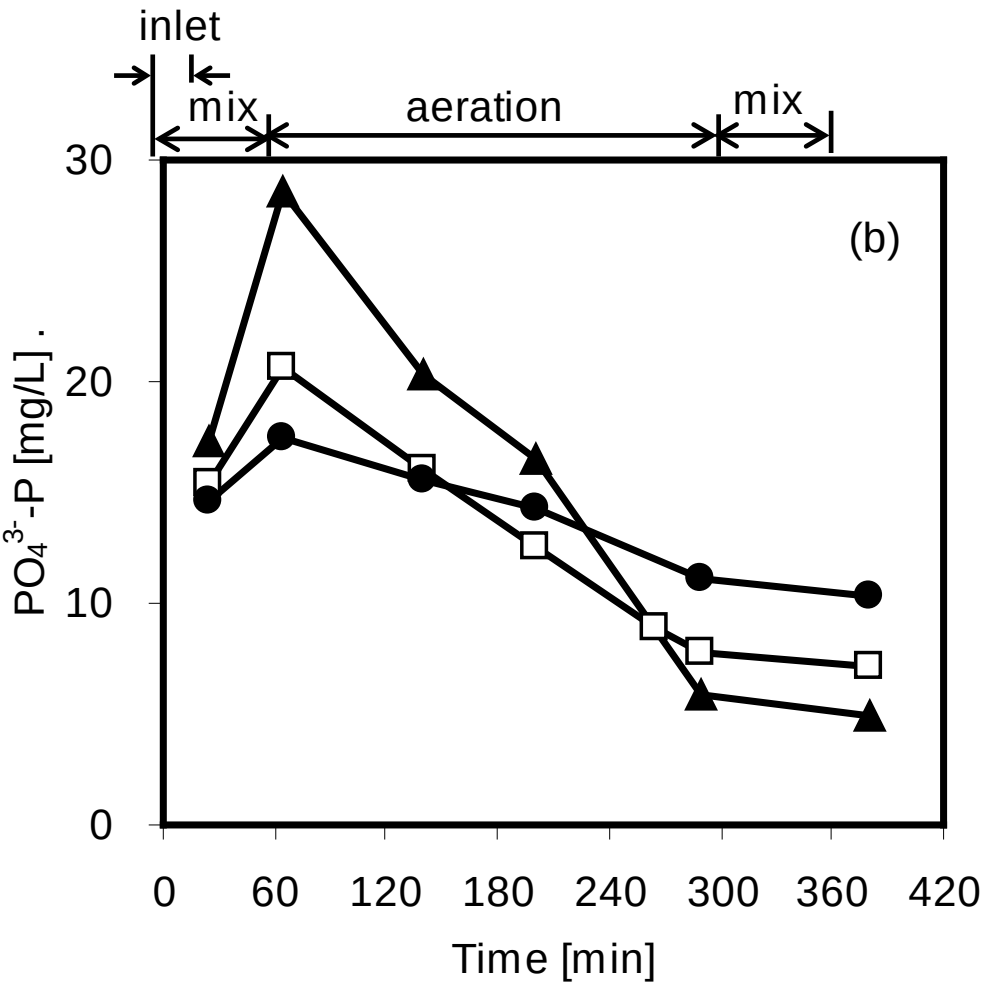


Fig. 3.5 a The averages of soluble PO_4^{3-}–P in the daily effluents of R1 (ERY-H_2O), R2 (ERY), and R3 (control).: soluble PO_4^{3-}–P of R1 (▲), R2 (□), and R3 (●)



Continued Fig. 3.5 b Comparison of phosphorus dynamics within the cycles of R1, R2, and R3 on day 160 during the steady states: soluble PO_4^{3-}–P of R1 (▲), R2 (□), and R3 (●)

3.4.4 PhyloChip-analyzed changes of microorganisms related to phosphorus and nitrogen removal

To further understand the slightly improved PO_4^{3-}–P removal in R1, genomic DNA extracted from steady state R1, R2, and R3 on day 367 was analyzed on the PhyloChips with probes that target the OTUs of *Rhodocyclus*-related poly-P accumulating organisms (PAOs) under the family of *Rhodocyclacea* and target the OTUs of glycogen accumulating organisms (GAOs, competing with PAOs for carbon

source) under the order of GAO cluster, respectively. With PAOs and GAOs in R3 as the control, results showed that the PAOs fluorescence intensity decreased and the GAOs intensity increased slightly in R1 (ERY-H_2O); both intensities of PAOs and GAOs in R2 (ERY) increased (Fig. 3.6). Also, the diversity of PAOs was lost in both R1 and R2 when comparing with that of R3 (Table 3.1). Therefore, ERY or ERY-H_2O changed phosphorus removal populations, which had potential impact on the phosphorus concentrations in the effluent. Moreover, due to limited OTUs related to PAOs and GAOs on the PhyloChip, the increase of GAOs intensity in R1 and R2 could not indicate the enhancement of GAOs by ERY or ERY-H_2O; similarly, the decrease of PAOs diversity and intensity in R1 might not represent the actual resistant PAOs selected by ERY-H_2O. The data are not enough to explain the phenomena of the slightly improved phosphorus removal in R1 (than R2 and R3).

In addition to phosphorus removal bacteria, nitrifying bacteria is another key group of organisms in wastewater treatment processes. The probes on the PhyloChip cover most of the nitrifying bacteria existing in WWTPs, including the AOMs under the family of *Nitrosomonadaceae* (including *Nitrosomonas*, *Nitrosospira*) and *Chromatiaceae* (*Nitrosococcus oceani*), the NOBs under the family of *Bradyrhizobiaceae* (*Nitrobacter*) and *Nitrospiraceae* (*Nitrospira*), and the anammox bacteria (strictly anaerobic bacteria occupying minor population in SBRs with aerobic conditions) under the family of *Anammoxales*, *Pirellulae*, and *Planctomycetaceae*. All these nitrifying bacteria studied are known as Gram-negative. Comparing with those in R3 (control), the fluorescence intensity of AOMs and NOBs decreased significantly in R1 (ERY-H_2O) and R2 (ERY), but anammox bacteria increased slightly in R1 and R2 (Fig. 3.6). The PhyloChip data also demonstrated taxa diversity reduction for nitrifying bacteria in R1 (50 µg/L of ERY-H_2O) and R2 (100 µg/L of

ERY) comparing with R3 (control; Table 3.1). The observed taxa diversity loss was the most for *Nitrosomonadaceae* (the dominant AOMs in WWTPs) in R1 of 80% and in R2 of 70%. The existing OTUs of *Nitrosomonadaceae* in R1 and R2 had the higher specific intensity than those in R3, suggesting that the less diverse AOMs selected by ERY-H_2O or ERY could play the same role as the more diverse community in R3. Indeed, ammonium oxidation was similar in the three SBRs as shown in Fig. 3.2, Fig 3.3 and Fig. 3.4. Similarly, nitrifying bacteria (AOMs and NOBs) with the same nitrification performance (Fig. 3.2, Fig. 3.3 and Fig. 3.4) but the lower diversity and total intensity in R1 and R2 than those in R3 (Table 3.1 and Fig. 3.6) may have a higher specific nitrification capacity which suggests a higher antibiotic resistance. For instance, the total intensity of AOMs on the PhyloChip showed a trend of R1 (ERY-H_2O)<R2 (ERY)<R3 (control), indicating that the specific ammonium oxidizing capacity and antibiotic resistance of AOMs were in a reverse order of R1 (ERY-H_2O)>R2 (ERY)>R3 (control). Similarly, the total intensity of NOBs demonstrated a trend of R2 (ERY)<R1 (ERY-H_2O)<R3 (control), indicating that the specific nitrite oxidizing capacity and antibiotic resistance of NOBs could be R2 (ERY)>R1 (ERY-H_2O)>R3 (control).

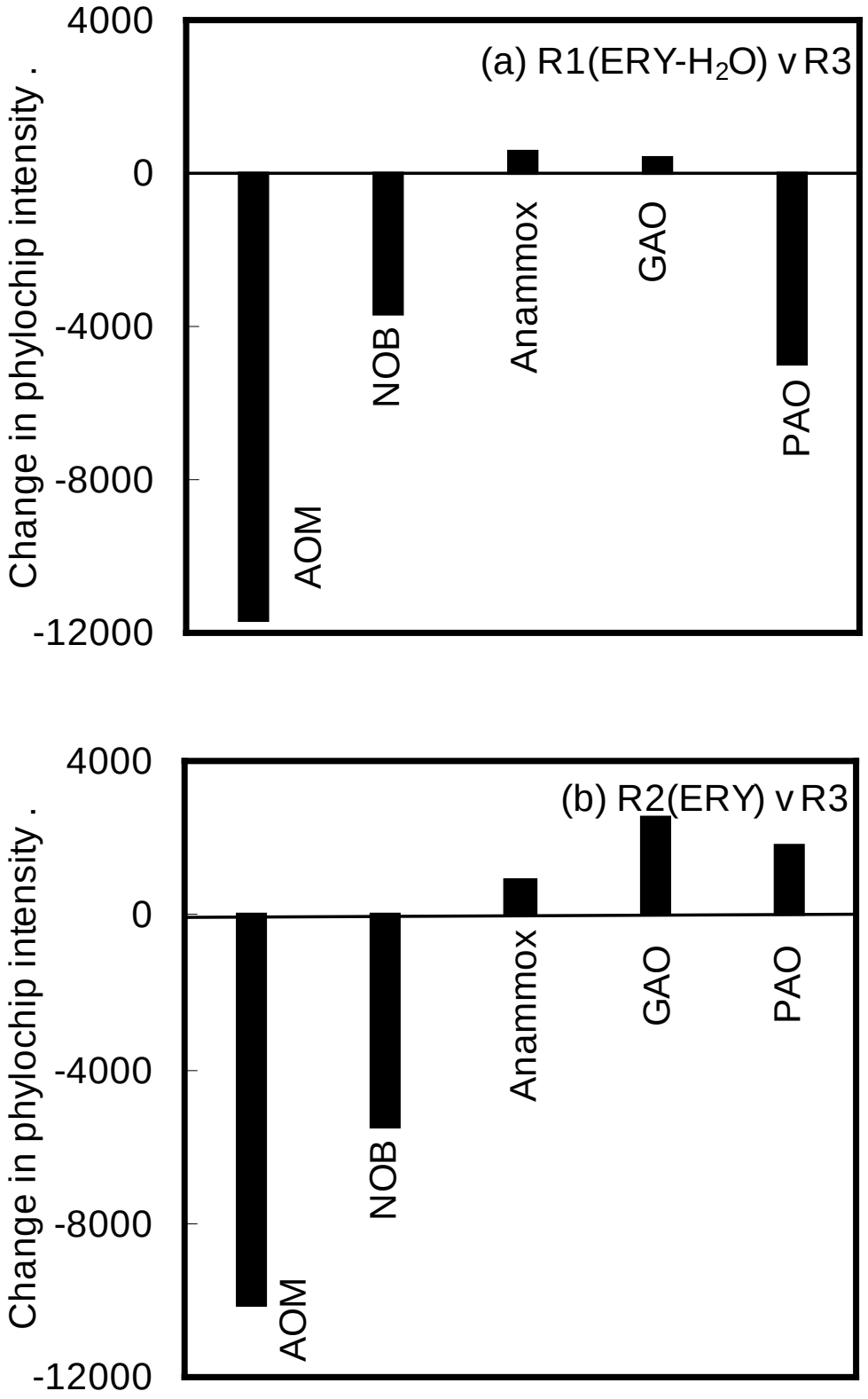


Fig. 3.6 PhyloChip analysis of microorganism populations related to nitrification and biological P removal in three steady state SBRs. *Bars above* the *zero line* represent bacteria that increased in abundance relative to R3; *bars below* represent those bacteria that declined in abundance

Table 3.1 PhyloChip analysis of microorganism diversity related to nitrification and biological P removal in three steady-state SBRs

	OTU richness relative to R3 (%)		Common OTUs relative to R3 (%)			
	R1	R2	R12	R13	R23	R123
AOMs-*Nitrosomonadaceae* (*Nitrosomonas*, *Nitrosospira*)	20	30	20	20	20	20
AOMs-*Chromatiaceae* (*Nitrosococcus oceani*)	71	57	43	71	57	43
NOBs-*Bradyrhizobiaceae* (*Nitrobacter*)	86	91	86	86	91	86
NOBs-*Nitrospiraceae* (*Nitrospira*)	50	100	50	50	100	50
Anammox bacteria	89	78	78	89	78	78
GAOs	100	133	100	100	100	100
PAOs	73	80	53	53	73	53

Relative to R3, common OTUs in R1 and R2 (R12); R1 and R3 (R13); R2 and R3 (R23); and R1, R2, and R3 (R123)

3.4.5 Resistance selection of nitrifying bacteria upon exposure to ERY or ERY-H_2O

To verify the resistance selection of nitrifying bacteria indicated by PhyloChip-observed shifts in AOMs and NOBs, biomass from the three steady state SBRs on day 390 were inoculated to the conical flasks with organic carbon free media spiked with different dosages of ERY or ERY-H_2O. The impact of biomass growth on the batch experiments can be ignored, since the optical densities of mixing liquid in the flasks showed no difference with time in the 3-day batch experiments. The negligible growth of the biomass can be explained by that fast growing heterotrophic bacteria cannot be supported by these organic carbon free media and the autotrophic nitrifying bacteria (usually occupy 2–3% of total activated sludge biomass) possess relatively long doubling time (such as >8 h at optimal conditions) (Koch et al., 2001; Prosser, 1989). In the flasks spiked with ERY-H_2O (at concentrations of 100, 400, and 800 µg/L), the biomass from each SBR completely oxidized ammonium or nitrite at similar rates as that of control (without ERY-H_2O spiked) within 3 days (data not shown), indicating that ERY-H_2O did not inhibit ammonium oxidation or nitrite oxidation in the short-term experiments, although ERY-H_2O (50 µg/L in R1) caused microbial community shift in the long-term operation.

Different from ERY-H_2O, ERY (at concentrations of 100, 400, and 800 µg/L) inhibited the whole nitrification process, and the inhibition extent depended on the biomass–source and the ERY concentration. For ammonium oxidation, significant difference of ERY inhibition was observed between biomass from R1 (ERY-H_2O) and R2 (ERY) (p<0.01), R1 (ERY-H_2O) and R3 (control; p<0.05) based on the statistical analysis of the 3-day product concentrations in the batch experiments. For nitrite oxidation, difference of ERY inhibition was obvious on biomass from R1

(ERY-H_2O) and R3 (control; p<0.01), but less obvious on biomass from R2 (ERY) and R3 (control) (p=0.07). In addition, the difference of ERY inhibition on nitrification processes was studied in detail on the 2rd incubation day by using inhibition percentage (for the same biomass, $100\% \times \left(1 - \frac{NO_x - N \text{ produced with ERY}}{NO_x - N \text{ produced without ERY}}\right)$). The maximum inhibition of ammonium oxidation by ERY (100, 400, and 800 µg/L) occurred on the biomass from R3 (control; 82–93%), followed by R2 (ERY; 71–95%) and R1 (ERY-H_2O; 56–91%) after 48 h reaction (Fig. 3.7), indicating that the resistance of AOMs to ERY was in an order of R1 (ERY-H_2O)>R2 (ERY)>R3 (control). Similarly, the maximum inhibition of nitrite oxidation by ERY (100, 400, and 800 µg/L) also occurred on the biomass from R3 (control; 33–61%) after 48 h reaction, but ERY inhibited the nitrite oxidation similarly on the biomass from R1 (ERY-H_2O; 21–42%) and R2 (ERY; 18–23%; Fig. 5), indicating that the resistance of NOBs to ERY was in an order of R2 (ERY)≥R1 (ERY-H_2O)>R3 (control). The results also demonstrate that ERY inhibited nitrite oxidation (18–61%) less significantly than ammonium oxidation (56–95%), which is consistent with the less NOBs diversity loss than AOMs as indicated by the PhyloChip readings (Table 3.1).

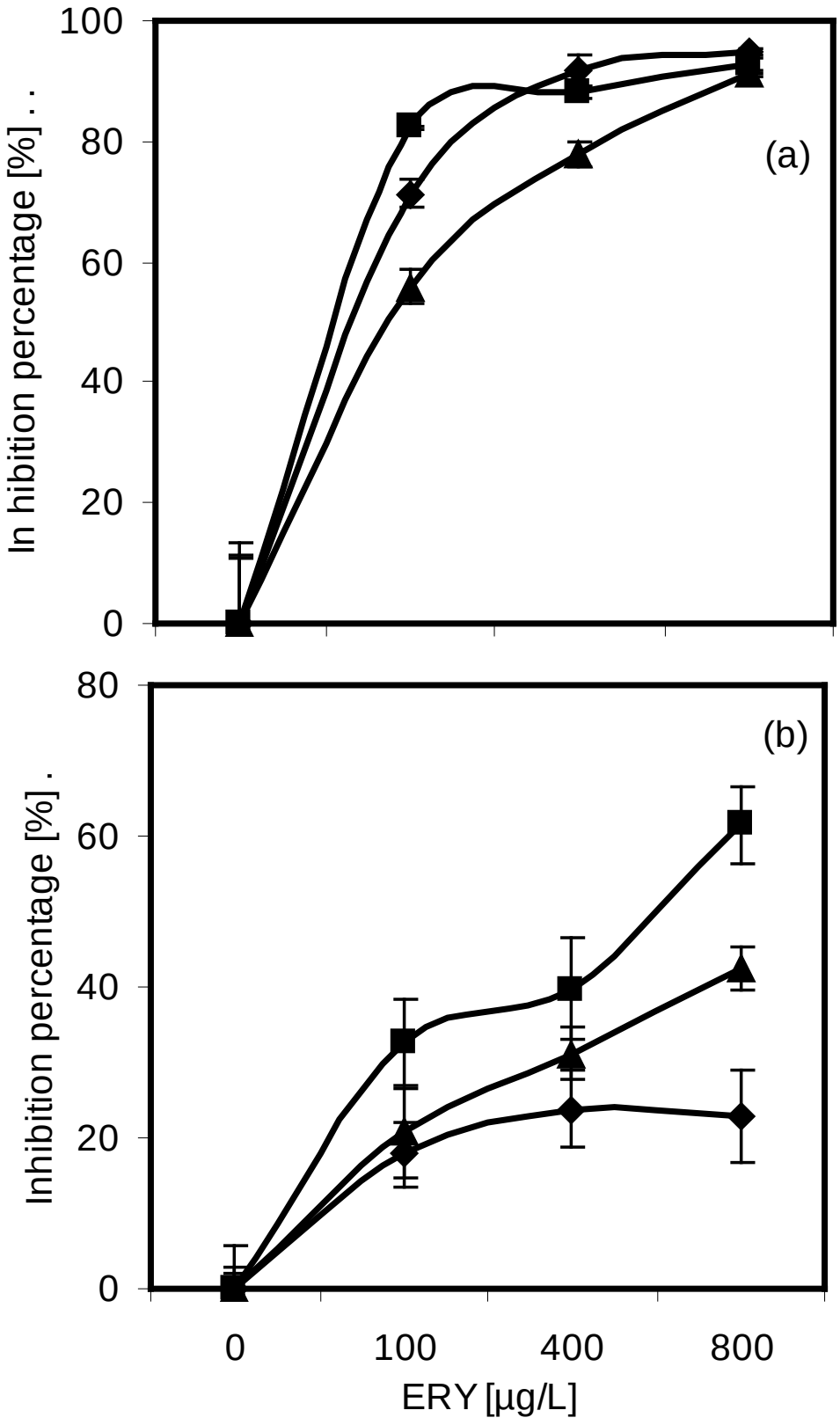


Fig. 3.7 In the batch experiments, **a** ammonium oxidation affected by ERY—(NO_2^-–N + NO_3^-–N) produced in the batches of R1 (ERY-H_2O; ▲), R2 (ERY; ◆) and R3 (control; ■) after 48 h incubation; and **b** nitrite oxidation affected by ERY—NO_3^-–N produced in the batches of R1 (ERY-H_2O; ▲), R2 (ERY; ◆) and R3 (control; ■) after 48 h incubation. The values represent means±standard deviations (n=3)

3.5 Discussion

In this study, a relatively low concentration of ERY (100 µg/L) or dehydrated ERY (ERY-H_2O; 50 µg/L) was investigated on their influences to the performance of the SBRs. Both ERY and ERY-H_2O showed negligible influence on carbon and nutrients (N and P) removal in the SBRs, which is consistent with the findings of the previous studies on even much higher concentrations of ERY (in mg/L range) (Amin et al., 2006; Oleinick, 1975). In contrast, analyzed on high-density microarrays (PhyloChip), bacteria related to N and P removal changed in both diversity and abundance (reflected by fluorescence intensity) due to exposure to ERY-H_2O (50 µg/L) or ERY (100 µg/L). The above observations could be explained by the selection of resistant bacteria, which are highly possible to be present in the inoculated activated sludge consisting of high diversity and density of bacteria (Kummerer, 2009a). This could lead to a shorter adaptation time of bacteria against antibiotics via resistance, such as the ability to biodegrade antibiotics or the efflux of antibiotics to eliminate toxic effects.

The resistant nitrifying bacteria selected upon exposure to low concentrations of the antibiotic ERY or its metabolite ERY-H_2O was further confirmed in the short-term batch experiments with higher concentrations of ERY at 100, 400, or 800 µg/L. This is one step further to answer the current open question about the resistance selection due to low concentrations of antibiotics in water by experimental data rather than by speculation (Hirsch et al., 1999). Previous studies reported that ERY-H_2O can induce Gram-positive *Staphylococcus aureus* RN 1389 to resist ERY (Majer, 1981). The present study demonstrates that ERY-H_2O can also induce nitrifying bacteria, which are all known as Gram-negative bacteria, to resist ERY. The biomass-

source dependent inhibition of ammonium oxidation by ERY (R1 (ERY-H_2O)<R2 (ERY)<R3 (control)) suggests that AOMs acclimated to ERY-H_2O (50 µg/L in R1) can create stronger resistance to ERY than AOMs acclimated to ERY (100 µg/L in R2). Also, the biomass-source dependent inhibition of nitrite oxidation by ERY (R2 (ERY)≤R1 (ERY-H_2O)<R3 (control)) suggests that NOBs acclimated to ERY-H_2O (50 µg/L in R1) can also create resistance to ERY. This shed light on the problem that formation of antibiotic metabolites does not mean elimination of environmental impact of antibiotics. In fact, some metabolites of antibiotics are more toxic to human than their parent drugs, such as acetyl derivative of sulfamethoxazole (Kummerer, 2009a). Additionally, current batch studies showed higher inhibition of ammonia removal (56–95%) and nitrate production (18–61%) by ERY (100, 400, and 800 µg/L; Fig. 3.7) than previous inhibition study on ammonium oxidation (0.20%) and nitrate production (0.28%) at ERY of 1,000 µg/L (Nimenya et al., 1999). The inhibition differences could be explained by different experimental conditions, such as Nimenya (1999) used shorter reaction duration, higher concentrations of nitrifying bacteria growing on the biofilms, and ERY solution prepared in deionised water at pH 5.8.

Different from nitrifying bacteria, the PhyloChip-observed information on PAOs and GAOs seems insufficient to explain the phenomena of the slightly improved phosphorus removal in R1 (than R2 and R3), which is mainly due to that PAOs and GAOs cover more complex but still largely uncertain genotypes than *Rhodocyclus*-related PAOs and GAO cluster (Seviour et al., 2003). Therefore, more information is needed in the future study.

In addition to the influence of ERY and ERY-H_2O on SBRs, the fate of these antibiotics in the reactors is another concern. As researchers suggested, the removal

of ERY or ERY-H_2O in WWTPs is highly possible due to the sorption by high concentrations of suspended sludge (Giger et al., 2003; McArdell et al., 2003). However, the removal efficiency of ERY-H_2O (70%) in R1 of this study was higher than those observed in the WWTPs at about 9–19% (Gulkowska et al., 2008) or ~ 50% (Yang et al., 2006). Besides possible biodegradation, the difference in sorption may be due to the different characteristics of the activated sludge. The SBRs in this study were fed with synthetic wastewater containing only soluble and easily degradable glucose as organic carbon source, while the real WWTPs contain complex organic carbon source. The different carbon sources could lead to the activated sludge with different extracellular polymeric substances, which would affect the sorption ability (Li and Yang, 2007). In addition, the degradation of ERY is another concern during studying its effects on the nutrients removal. Since neither ERY nor ERY-H_2O was detected in the effluent of R2 (ERY) and biosorption has never been reported to have such high removal efficiency on ERY or ERY-H_2O at even lower concentrations, ERY was suspected to be transformed to products other than ERY-H_2O. Our discovery is different from previous studies in which the unchanged ERY was degraded easily to ERY-H_2O in the aquatic environment (Giger et al., 2003; Hirsch et al., 1999; Karthikeyan and Meyer, 2006; Kolpin et al., 2002; McArdell et al., 2003; Richardson et al., 2005; Xu et al., 2007b). The difference may be due to various pH values in the studies or may be due to biodegradation of ERY. Further investigation is needed to identify the degradation mechanism of ERY and the degradation product(s).

3.6 Conclusions

In conclusion, ERY-H_2O (50 µg/L) and ERY (100 µg/L) exhibit no unfavorable results on the efficiency of SBRs in treating the synthetic wastewater.

However, both compounds cause changes of microbial community composition in the reactors, which leads to the selected microorganisms more adaptive and resistant to the inhibitory effects of ERY and ERY-H_2O. This study suggests that the influence of ERY and ERY-H_2O at the μg/L levels on treatment systems such as the complex wastewater treatment plants, one of the most highly antibiotics-exposed environments, is more likely to induce a pool of ERY resistance genes than to inhibit the treatment.

Chapter 4

Proliferation of Antibiotic Resistance Genes in Microbial Consortia of SBRs upon Exposure to Trace ERY or ERY-H_2O

4.1 Abstract

A variety of antibiotics and their metabolites at sub-inhibitory level concentrations are suspected to expand resistance genes in the environment. However, knowledge is limited on the causal correlation of trace antibiotics or their metabolites with resistance proliferation. In this study, ERY resistance genes were screened on microbial consortia of SBRs after one year acclimation to ERY (100 µg/L) or ERY-H_2O (50 µg/L). The identified esterase gene *ere*A explains that ERY could be degraded into six products by microbes acclimated to ERY (100 µg/L). However, ERY could not be degraded by microbes acclimated to ERY-H_2O (50 µg/L), which may be due to the less proliferated *ere*A gene. Biodegradation of ERY required the presence of exogenous carbon source (e.g., glucose) and nutrients (e.g., nitrogen, phosphorus) for assimilation, but overdosed ammonium–N (>40 mg/L) inhibited degradation of ERY. *Zoogloea*, a type of biofilm-forming bacteria, became predominant in the ERY degradation consortia, suggesting that the input of ERY could induce biofilm resistance to antibiotics. Our study highlights that lower µg/L level of ERY or ERY-H_2O in the environment encourages expansion of resistance genes in microbes.

4.2 Introduction

Antibiotics are detected in the environment such as hospital effluent, municipal wastewater, surface water, and groundwater (Kummerer, 2009a). The most frequently detected antibiotics and their derivatives include trimethoprim, tetracycline, norfloxacin, penicillin G, cefalexin, cefotaxim, and dehydrated erythromycin (ERY-H_2O), which are usually persistent in WWTPs (Kummerer, 2009a). The persistence of antibiotics may accelerate the development of resistance

genes and resistance bacteria by exerting selective pressure on microbes in the environment. The occurrence and transfer of new combination of resistance genes are predicted to be much more frequent in the compartments with higher diversity and abundance of microorganisms (Murray, 1997), such as WWTPs that receive various antibiotics and their metabolites discharged from residential or hospital areas (Gros et al., 2007). So far, it is not clear and far from complete whether antibiotics at concentrations as low as detected in hospital effluents (in the higher µg/L range) or in the aquatic environment (in the lower µg/L range) are important for the expansion of resistance in microbes (Kummerer, 2009b). The correlation of input of antibiotics at lower environmental concentrations with the development or occurrence of antibiotic resistance genes is short of support with experimental data (Kummerer, 2009b). Other findings indicate that continuous input of resistant bacteria and resistance genes rather than the presence of antibiotics at sub-inhibitory concentrations may be more important for keeping resistance in the environment (Ohlsen et al., 2003; Ohlsen et al., 1998). All these uncertainties have inspired researchers to investigate the fate of antibiotics and the occurrence of resistance in WWTPs and downstream natural water regions in the past decade (Le-Minh et al., 2010). However, these investigations have been becoming increasingly difficult due to the lack of reference WWTPs free from input of resistance bacteria and genes. Without a proper reference from an environmental system against resistance input, influence of antibiotics on resistance proliferation is inconclusive.

Among the antibiotics studied, ERY has received less attention compared with other antibiotics in WWTPs since ERY is sensitive to pH. At the operational pH ranges (6.5–8) of most municipal WWTPs, active ERY co-exists with its dehydrated-form ERY-H_2O (Le-Minh et al., 2010). Erythromycin-H_2O is removed

mainly by sorption to sewage sludge in WWTPs via hydrophobic interactions and cation exchanges due to its surfactant-like structure, but its lower removal efficiency (9–19%) results in its higher concentration up to 6 µg/L in the effluent of WWTPs (Kummerer, 2009a; Le-Minh et al., 2010). Moreover, ERY-H_2O was reported to induce bacterial resistance as ERY does (Fan et al., 2009; Majer, 1981). Accordingly, the occurrence of ERY derivatives other than ERY-H_2O could also be possible to introduce microbial resistance. Up to now, knowledge is still scarce on the contribution of ERY or other ERY derivatives (e.g., ERY- H_2O) at µg/L levels in wastewater treatment systems to the amplification of resistance genes.

Microbial resistant mechanisms to ERY include excretion of ERY by efflux pumps (e.g., efflux genes *mef*A/E and *msr*A/B), alteration of the target site to avoid binding of ERY (e.g., erythromycin ribosomal methylase genes *erm*), and destruction of ERY directly (e.g., esterase genes *ere*A and *ere*B, macrolide-2'-phosphotransferase gene *mph*A) (Amin et al., 2006; Wright, 2005). Among these resistance genes, *erm* genes (A, B, C, E, F, T, V, and X), *mef* genes (A, E, and I), *msr*A, *ere*A/B, and *mph*A genes have been detected in wastewater and activated sludge of WWTPs, and *erm*B is the most prevalent gene in the environmental samples (Szczepanowski et al., 2009; Zhang et al., 2009). Actually, all modes of ERY-related resistance genes can be found in both Gram-positive and Gram-negative bacteria (http://www.ncbi.nlm.nih.gov/), but intrinsic possession of efflux resistance on Gram-negative bacteria's membrane results in less sensitivity of them to ERY than Gram-positive bacteria (Pechere, 2001). Another mode of ERY resistance, destruction or the so-called biodegradation of antibiotics can be used as an indicator to interpret the function of antibiotic resistance genes. Until recently, a few studies have tackled the possible biodegradation of ERY at different concentrations (Alexy et al., 2004;

Gartiser et al., 2007b). A closed bottle test exhibited that ERY at concentrations of 2.46 mg/L (equal to the theoretical oxygen demand (ThOD) of 5 mg/L) was not readily biodegradable by activated sludge (Alexy et al., 2004). Erythromycin at 167 mg /L (equal to TOC of 100 mg/L) could not be degraded completely and had evident inhibition on carbon removal (Gartiser et al., 2007b). Noteworthy, the inocula utilized by the above studies were taken from WWTPs not receiving effluents from hospitals and were assumed to be less-adapted to antibiotics. Biodegradation of antibiotics would rarely occur by non-selected microbes, that is, in the absence of resistance genes (Ding and He, 2010; Kummerer, 2009b). In order to highlight effects of low concentrations of ERY and ERY- H_2O on amplification of resistance genes, it is of significance to investigate biodegradation of ERY by microorganisms acclimated to ERY or ERY-H_2O (in the µg/L range).

The aim of this study is to identify the development of resistance genes and to investigate biodegradation of ERY with microbial consortia that have been acclimated to ERY (100 µg/L) or ERY-H_2O (50 µg/L) for over one-year running with synthetic wastewater free from resistant bacteria and resistance genes input. Findings of this study will provide significant information for the inadequate data on effects of trace antibiotics to promote resistance genes development in the aquatic and terrestrial environment.

4.3 Materials and methods

4.3.1 Batch experiments

Biodegradability of ERY (10 mg/L, simulation of peak concentration in the effluent of pharmaceutical production plants) was tested in 250 ml flask bottles with inocula from steady state R1 (ERY-H_2O of 50 µg/L), R2 (ERY of 100 µg/L), and R3

(control) after running over one year (the startup, operation methods and performance data of the three SBRs were described in chapter 3). The inocula were freshly applied to short-term batch experiment. Before being inoculated to flask bottles, the activated sludge of 1 ml withdrawn from the three steady state SBRs were washed three times with the same medium used in the batches. Negative controls were prepared with autoclaved sludge to indicate possible ERY removal by non-biotic processes. Bottles were filled with 50 ml of medium similar to the synthetic wastewater described above except for various concentrations of COD (contributed by glucose), NH_4^+–N, and PO_4^{3-}–P in different batches as described below (Table 4.1). (1) Effects of inocula source on degradation of ERY: ERY degradation capability was tested with inocula from R1 (ERY-H_2O), R2 (ERY), and R3 (control) by using medium containing COD, NH_4^+–N, PO_4^{3-}–P, and ERY of 600, 30, 6, and 10 mg/L (COD:N:P = 100:5:1, as assimilation required), respectively. (2) Effects of glucose on degradation of ERY: ERY (10 mg/L) degradation by inoculum from R2 (ERY) was tested in medium with glucose equal to 600 mg/L COD or without glucose. Other elements in the medium were exactly the same as above mentioned (e.g., NH_4^+–N of 30 mg/L and PO_4^{3-}–P of 6 mg/L). (3) Effects of ammonium and phosphate on degradation of ERY: Degradation of ERY (10 mg/L) was tested with inocula from R2 (ERY) by using medium containing 600 mg/L COD, 6 mg/L PO_4^{3-}–P and gradient concentrations of NH_4^+–N (30, 40, 50, and 60 mg/L), or 30 mg/L NH_4^+–N and gradient concentrations of PO_4^{3-}–P (6, 13, 20, and 26 mg/L). All the above experiments were performed in triplicates. The flask bottles were placed on a shaker for mixing and aeration at room temperature (25–27°C). The pH ranged from 6.9 to 8.1 during one batch of experiments without adjustment. Samples from batch conical flasks were collected

daily and centrifuged at 14,000 rpm for 15 min at 25°C. Therefore, the measured phosphate and all kinds of nitrogen were in dissolved forms throughout this study.

Table 4.1 Batch experiments to study effects of inocula source, glucose (calculated as COD), NH_4^+–N, and PO_4^{3-}–P on biodegradation of ERY (10 mg/L)

Factors	Inocula source	COD (mg/L)	NH_4^+–N (mg/L)	PO_4^{3-}–P (mg/L)
Inocula source	R1, R2, and R3	600	30	6
Glucose	R2	600 and 0	30	6
Ammonium	R2	600	30, 40, 50, and 60	6
Phosphate	R2	600	30	6, 13, 20, and 26

4.3.2 Analytical methods

As described in chapter 3, an ion chromatography was used to measure dissolved PO_4^{3-}–P, NO_3^-–N, NO_2^-–N and NH_4^+–N, and a LC-MS-MS was applied to detect both ERY and ERY-H_2O. Products of ERY were full-scanned by LC-MS over the *m/z* range of 100–1000 amu.

4.3.3 DNA extraction and polymerase chain reaction (PCR)

The genomic DNA of 1 ml of mixed liquid from the SBRs was extracted and purified by using DNeasy Tissue Kit (QIAGEN GmbH, Germany) with modified method as described in chapter 3 (Fan et al., 2009). The obtained DNA was quantified on a Nanodrop-1000 (NanoDrop Technologies Inc., USA).

PCR (Eppendorf, Germany) amplification of 16S rRNA genes was performed by using universal eubacterial primers 8F (Zhou et al., 1995) and 1392R (Lane et al., 1985). The fluorescently labeled forward primer 8F-cy5 was used to amplify 16S rRNA genes for T-RFLP. Erythromycin resistance genes, including esterase genes

*ere*A and *ere*B, phosphotransferase gene *mph*A, efflux genes *mef*A/E and *msr*A/B, and methylase genes *erm*A, *erm*B, and *erm*C, were PCR (Eppendorf, Germany) amplified by using primers described previously (Sutcliffe et al., 1996). PCR products were subsequently visualized on a Molecular Imager Gel Doc XR System (Bio-Rad, USA).

4.3.4 T-RFLP

The fluorescently labeled 16S rRNA genes were digested with 5 U of restriction endonucleases *Hha*I, *Msp*I, or *Rsa*I (NEB, USA) as described previously (He et al., 2003). The terminal restriction fragments (T-RFs) were determined on a CEQ 8000 automated sequencer (Beckman Coulter, USA) by using GenomeLab™ Fragment Analysis Kit Insert (608113-AH) and internal standard (DNA Size Standard Kit – 600, p/n 608095) (Beckman Coulter, USA). Individual T-RFs were normalized as a percentage of the total peak area.

4.3.5 Clone library and sequencing

A 16S rRNA gene clone library was established by using TOPO-TA cloning kit (Invitrogen, USA) according to the manufacturer's recommendation. The 16S rRNA gene inserts were PCR-amplified with TA primers (Zhou et al., 1997) and then digested with enzyme *Hha*I or *Msp*I (NEB, USA). Plasmid DNA was purified with Qiagen Miniprep kit (Qiagen GmbH, Germany) and subsequently sequenced on an ABI 3100 Sequencer (Applied Biosystems, USA) by using primers M13F-20, M13R-24, 533F, 529R, 907F (http://www.genomics.msu.edu). Sequences were aligned and analyzed by using BioEdit assembly software

(http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and BLAST (http://www.ncbi.nlm.nih.gov/), respectively.

4.4 Results

4.4.1 Effects of ERY and ERY-H_2O on expansion of resistance genes

Erythromycin resistance genes were screened on microbial consortia in the SBRs (Table 4.2 and Fig. 4.1). Esterase genes *ere*A and *ere*B, efflux gene *mef*A/E, and methylase gene *erm*A were detected in the microbes of MR (before 8-month pretreatment). After 8-month pretreatment, *ere*A, *ere*B, and *erm*A genes disappeared in the MR and the latter two genes did not recover in any reactors regardless of the one year addition of ERY or ERY-H_2O; *mef*A/E always appeared at a lower level in the MR and kept in all three SBRs (R1, R2, and R3) in the following one more year operation. Interestingly, though *ere*A gene was not detected in the MR after 8-month pretreatment, it bounced back in R1 (ERY-H_2O) and R2 (ERY), but was not detected in R3 (control). No any other factors were different in the three SBRs except the presence/absence of ERY-H_2O or ERY. Thus, both ERY-H_2O (50 μg/L in R1) and ERY (100 μg/L in R2) exhibited significant effects on proliferation of the resistance gene *ere*A.

Table 4.2 Resistance genes detected in MR, R1 (50 µg/L of ERY-H_2O), R2 (100 µg/L of ERY) and R3 (control)

Gene	Function \ Month	-8	0	12	12	12
		MR	MR	R1(ERY-H_2O)	R2(ERY)	R3(control)
*ere*A	Erythromycin	+ [a]	— [b]	+	++ [c]	—
*ere*B	esterase	+	—	—	—	—
*mph*A	Macrolide-2'-	—	—	—	—	—
	phosphotransferase					
*mef*A/E	Macrolide efflux	++	+	+	+	+
*msr*A/B	Macrolide efflux	—	—	—	—	—
*erm*A	Erythromycin	—	—	—	—	—
*erm*B	ribosomal	+	—	—	—	—
*erm*C	methylase	—	—	—	—	—

Note: [a] + detected, [b] — not detected, [c] ++ relatively higher concentrations of PCR products detected.

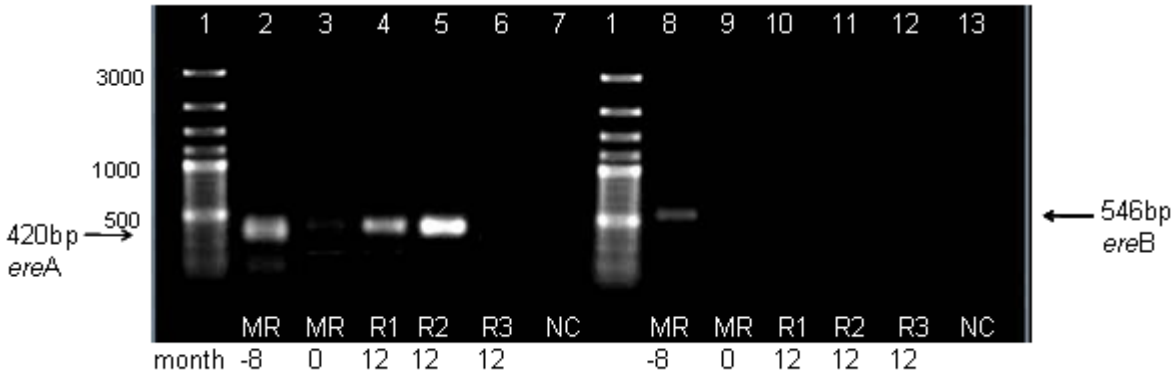


Fig. 4.1 Detection of esterase genes *ere*A and *ere*B in the microbes of mother reactor (MR), R1 (ERY-H_2O), R2 (ERY), and R3 (control). Lane 1, Generuler^{TM} 100 bp Plus ladder (Fermentas); lanes 2-7, 420 bp PCR products of *ere*A in the microbes of MR (month -8 and 0), R1, R2 and R3 (month 12), and negative control (NC); lanes 8-13, 546 bp PCR products of *ere*B in the microbes of MR (month -8 and 0), R1, R2 and R3 (month 12), and NC.

4.4.2 Biodegradation of ERY

Microbial consortia containing esterase gene *ere*A may be capable of esterifying ERY. Therefore, microorganisms from R1 (ERY-H_2O), R2 (ERY), and R3 (control) were tested on their capability to degrade ERY (10mg/L) in batch bottles with a medium containing COD, NH_4^+–N, and PO_4^{3-}–P of 600, 30, and 6 mg/L (COD:N:P = 100:5:1, as assimilation required), respectively. A remaining percentage of ERY (concentration of ERY in the tested bottles compared to that in negative controls with autoclaved inocula) was used to indicate biodegradation of ERY. During five days incubation, remaining percentage of ERY was constantly ~ 100% in the batches with inocula from R1 (ERY-H_2O) or R3 (control), indicating that ERY-H_2O (50 µg/L in R1 running over one year) could not acclimate microbes to degrade ERY (Fig. 4.2). In contrast, ERY in batches with inocula from R2 (ERY) was completely removed within 3 days, indicating that ERY (100 µg/L in R2 running over one years) induced microbes to degrade ERY (Fig. 4.2). In addition, similar batches were setup to test biodegradation of ERY-H_2O, but results showed that ERY-H_2O was persistent regardless of the inoculum source (data not shown).

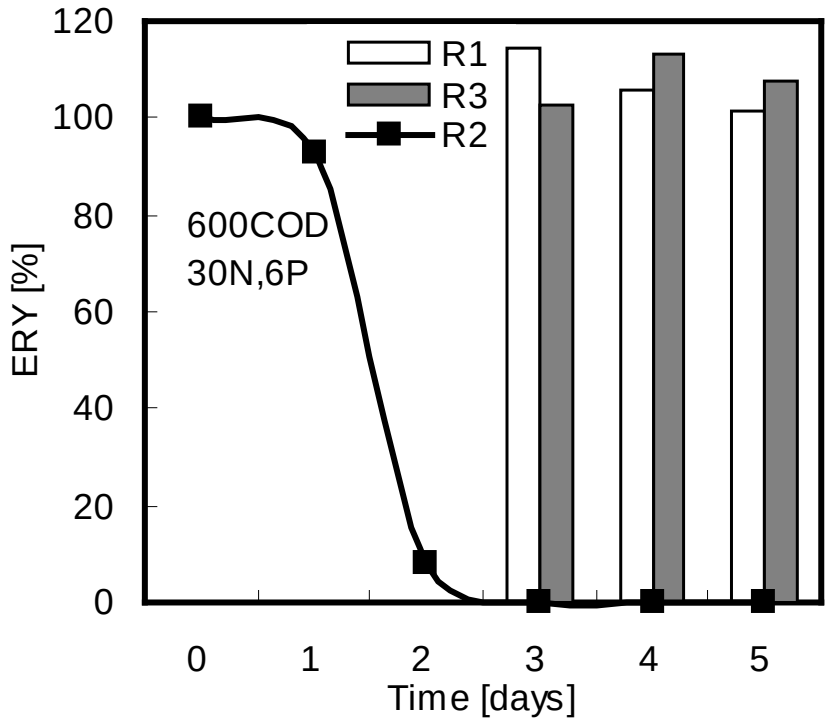


Fig. 4.2 Degradation of ERY in the batches with inocula from R1 (ERY-H_2O), R2 (ERY), and R3 (control). A percentage of ERY is determined by the concentration of ERY in the tested bottles compared to that in the negative control bottles with autoclaved inocula. The values represent an average (n=3), and the standard deviations (less than 6%) were not shown.

To investigate the degradation products of ERY, LC-MS-MS was used to monitor the precursor ion of ERY with a mass-to-charge ratio *m/z* 734.5/158.2 amu. The LC-MS-MS chromatograms demonstrated that two main peaks were eluted at a retention time of 5 min and 10 min with the disappearance of ERY in the batches with inocula from R2 (ERY at 100 µg/L), suggesting that at least two products (product I and II) with the same precursor ion *m/z* ratio as ERY were produced (Fig. 4.3a). Furthermore, biodegradation products of ERY were fully scanned using LC-MS (*m/z* 100-1000 amu), the chromatogram of which exhibited six products (Fig. 4.3b). According to the mass spectra of these six products, four products with the same precursor ion *m/z* ratio (735 amu) as ERY appeared at a retention time of 4.6 (product

II), 9.2, 9.6, and 10.3 min (product I); and the other two products with lower *m/z* ratios (718 amu and 720 amu) of precursor ions than ERY eluted at 9.9 min and 10.6 min, respectively (Fig. 4.4). The six products, possessing either the same or 15–17 amu lower *m/z* ratios of precursor ions (735, 720, and 718 amu) than ERY (*m/z* 735 amu), are good matches of the downstream products of esterified ERY as reported previously by isolates of *Escherichia coli*, *Providencia stuartii*, *Staphylococcus aureus*, and *Pseudomonas sp.* (Wright, 2005).

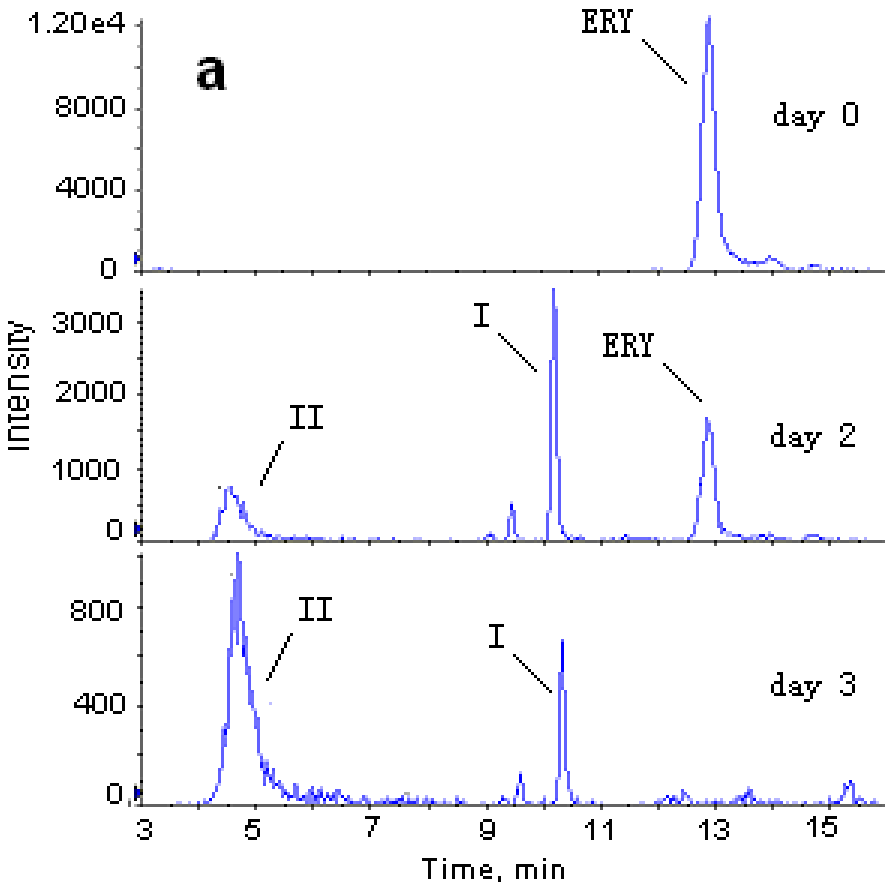
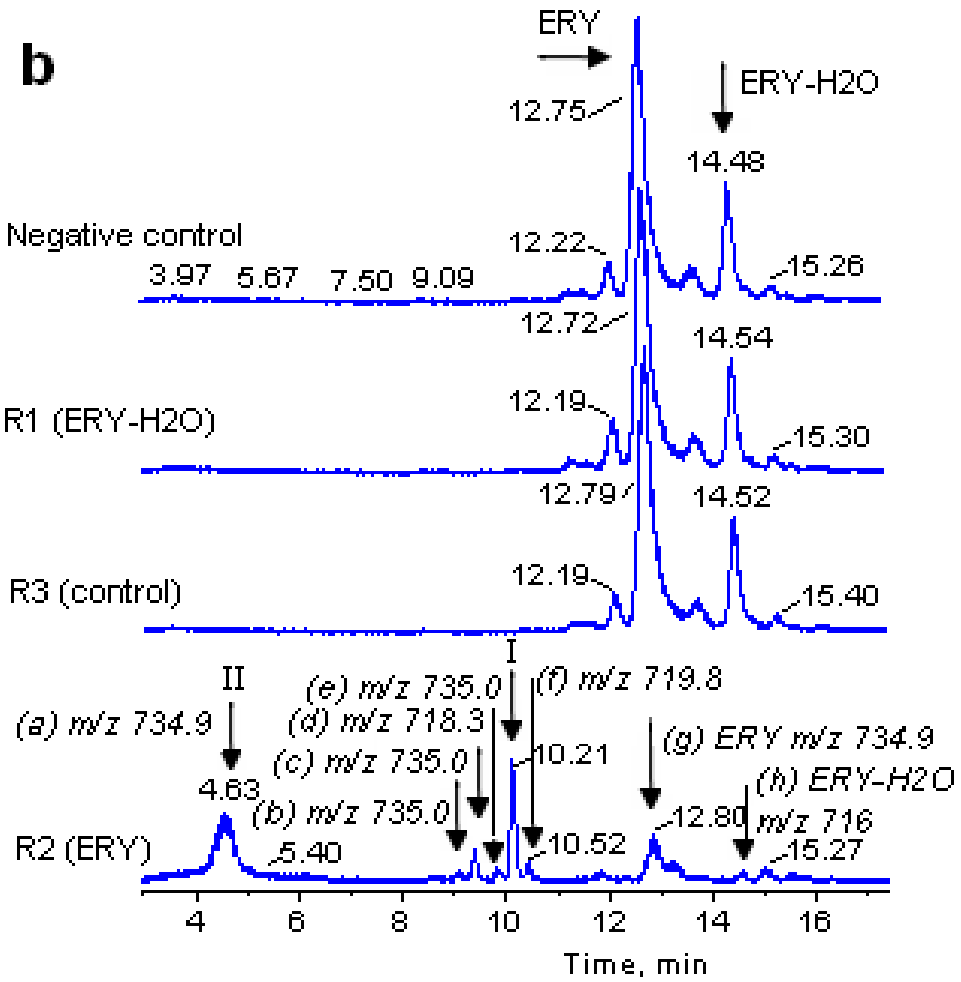


Fig. 4.3 Biodegradation products of ERY. **a —** The LC-MS-MS chromatograms (734.5/158.2 amu) exhibit the degradation products of ERY in the batches of R2 (ERY) (shown in Fig. 4.2) after incubation for 0 day, 2 days and 3 days.



Continued Fig. 4.3 Biodegradation products of ERY. **b —** The LC-MS chromatograms (full-scan with *m/z* 100–1000 amu) exhibit the degradation products of ERY (shown in Fig. 4.2) after incubation of 2 days.

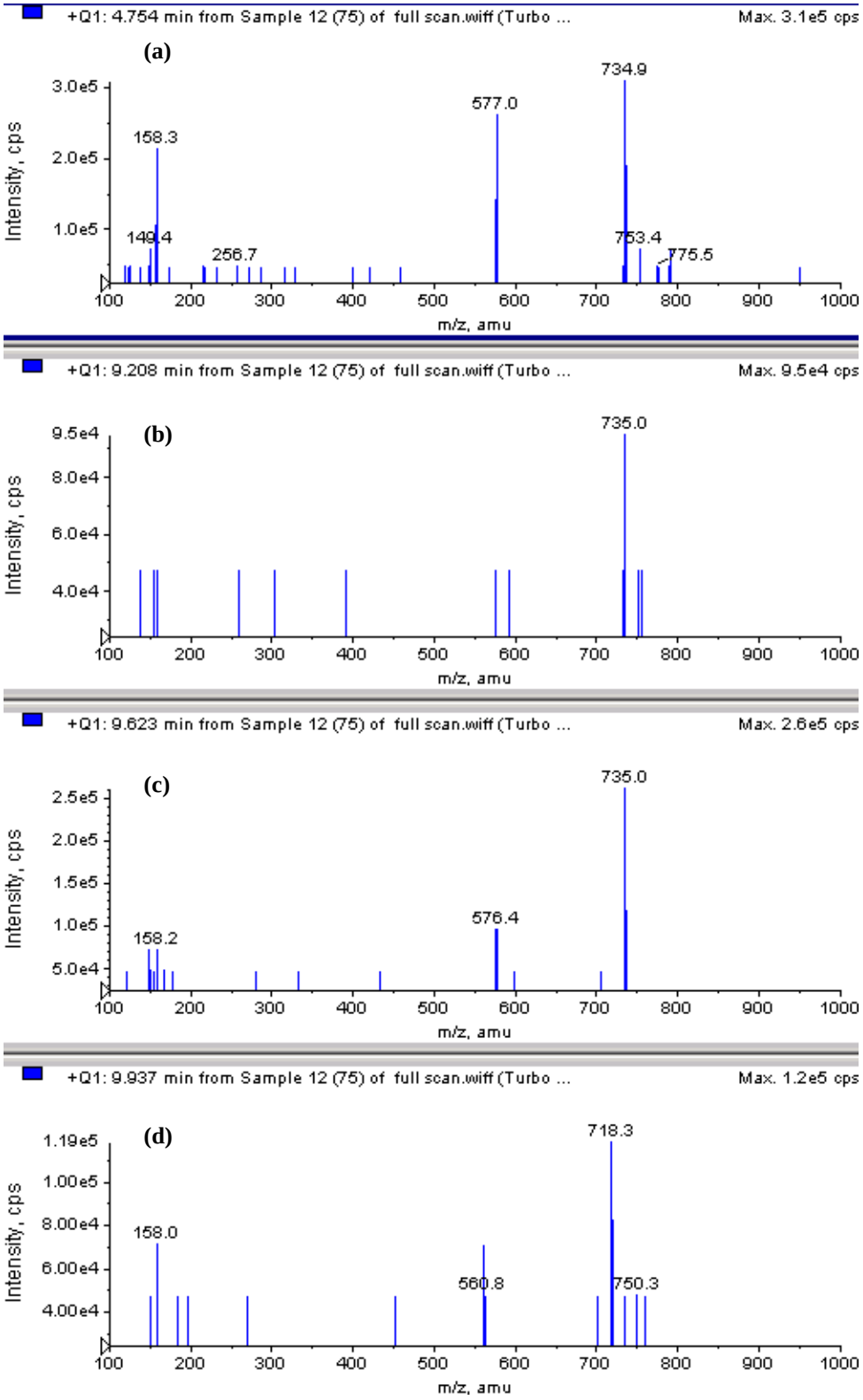
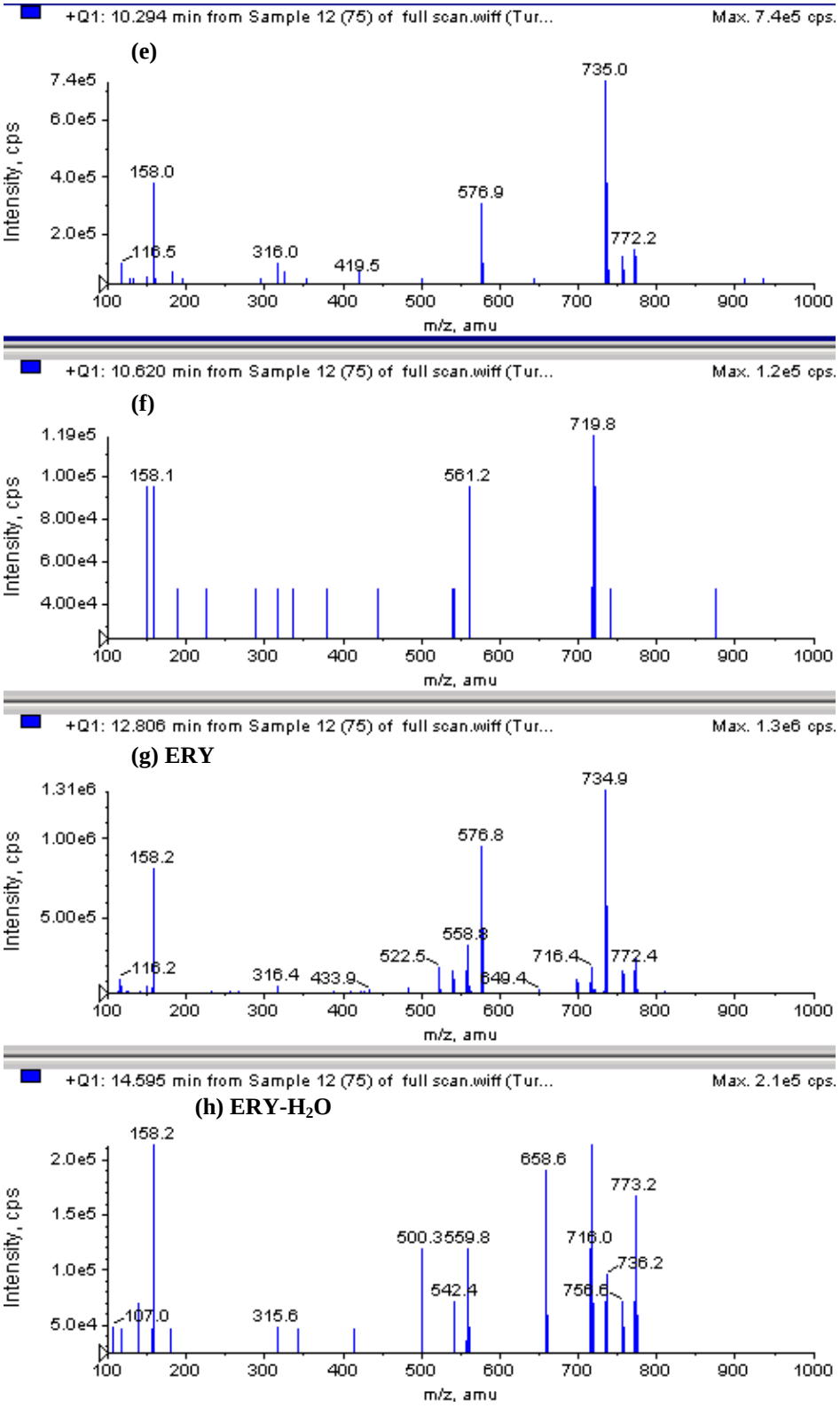


Fig. 4.4 Mass spectra of peaks in the Fig. 3b: peaks at retention time (a) 4.6 min (product I), (b) 9.2 min, (c) 9.6 min, and (d) 9.9 min.



Continued Fig. 4.4 Mass spectra of peaks in the Fig. 3b: peaks at retention time (e) 10.3 min (product II), (f) 10.6 min, (g) 12.8 min and (h) 14.5 min.

Based on the degradation products and previous reported esterase mechanism of ERY (Wright, 2005), the possible pathway for ERY biodegradation was shown in Fig. 4.5. The formation of two main products I and II (*m/z* 735 amu) from ERY may follow four steps: 1) esterase enzyme cleaves the macrocycle ester via adding one H_2O molecule, 2) non-enzymatic intramolecular hemiketal formation (one ERY –OH group transformed), 3) internal dehydration to form enol ether product I, or a second internal cyclization event via intramolecular condensation and dehydration to form product II (one ERY –OH group transformed), 4) Product I transferred to product II (one ERY –OH group transformed). The other two products with precursor ion *m/z* ratios (735 amu) may share the same formation mechanism with the products I and II at the first 2 steps, but differ in dehydration positions at the third step. The two products with precursor ion *m/z* ratios (720 and 718 amu) may be dehydrated twice at the third step (at most two ERY –OH groups transformed) and followed by saturation with several –H. Overall, biodegradation of ERY seemed to transform 1–3 –OH groups. As most of –OH groups of ERY are active groups for antibiotic activity (Schlunzen et al., 2001), the biodegradation products of ERY may lose or weaken their antibiotic effects as ERY-H_2O does.

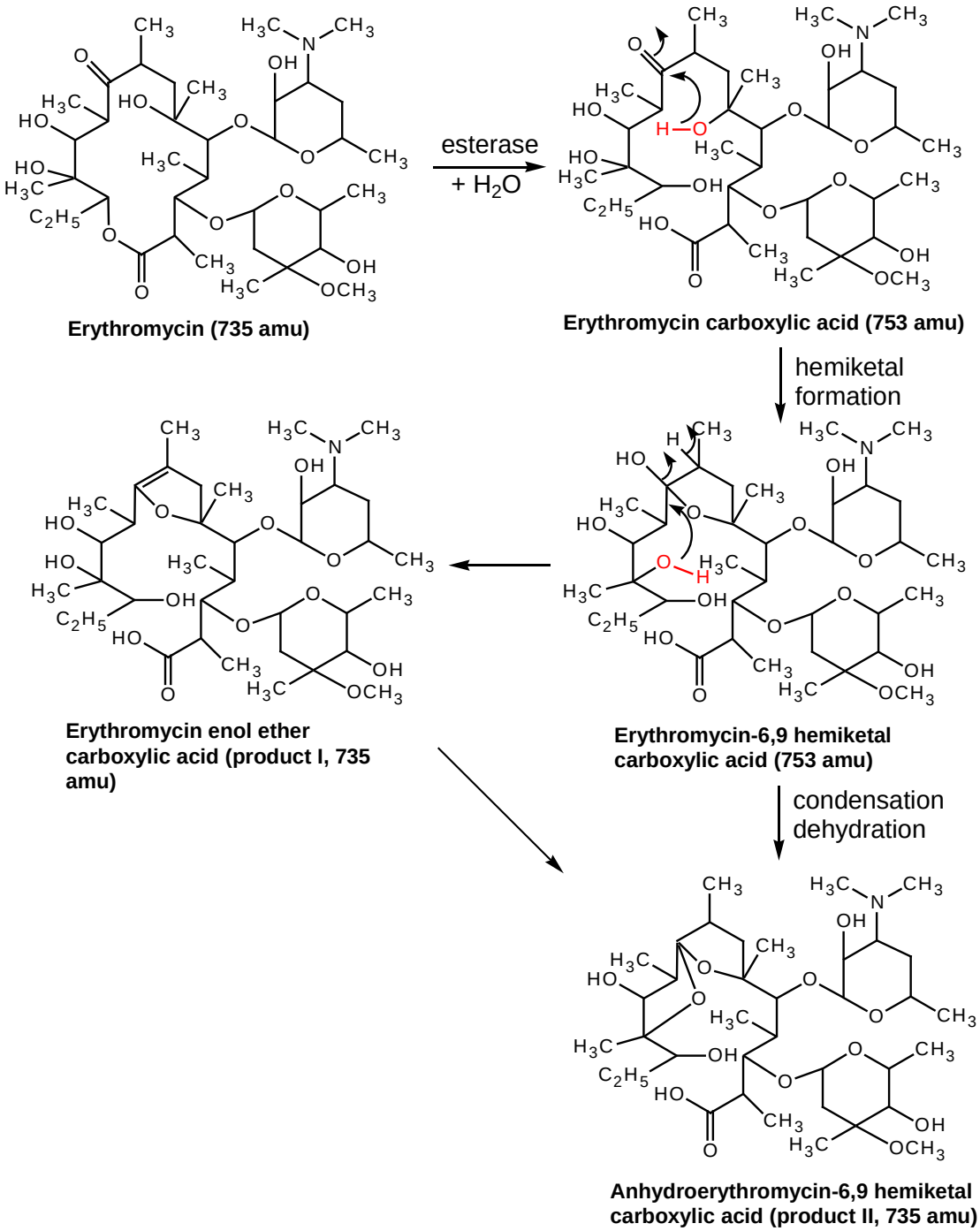


Fig. 4.5 Reaction and downstream products of ERY esterases.

4.4.3 Effects of glucose, ammonium and phosphate on biodegradation of ERY

Since most of ERY esterase-producing microbes, such as *E. coli* and *S. aureus* (Wondrack et al., 1996), are known as heterotrophic bacteria, it is necessary to assess the influence of exogenous carbon source (e.g., glucose) on ERY biodegradation. The

medium containing NH_4^+–N (30 mg/L) and PO_4^{3-}–P (6 mg/L) was prepared in two conditions with or without glucose (equal to COD of 600mg/L) and received inocula from R2 (ERY). In the medium without glucose, 10 mg/L ERY did not show any obvious decrease compared with negative control. In the medium containing glucose, however, ERY decreased quickly from 10 mg/L to ~1 mg/L within 3 days (Fig. 4.6a), suggesting that the exogenous carbon source (e.g., glucose) was necessary for degrading ERY.

To evaluate effects of ammonium and phosphate on ERY biodegradation, the medium containing different initial concentrations of NH_4^+–N (30, 40, 50, and 60 mg/L) and PO_4^{3-}–P (6, 13, 20, and 26 mg/L) was tested on inocula from R2 (ERY). In batches with different phosphate concentrations (NH_4^+–N of 30 mg/L and COD of 600 mg/L), ERY degradation was similar for all batches (Fig. 4.6b), excluding the effects of phosphate on the biodegradation of ERY. Ammonium, however, was shown to greatly inhibit the biodegradation of ERY (Fig. 4.6c). When inocula were fed with NH_4^+–N of 30 mg/L (theoretically just sufficient for assimilation of 600 mg/L COD), ERY of ~9 mg/L (90%) was biodegraded; whereas with higher concentrations of NH_4^+–N, ERY of 7 mg/L (70%, NH_4^+–N of 40 mg/L), and 3 mg/L (30%, NH_4^+–N of 50 and 60 mg/L) was biodegraded, respectively.

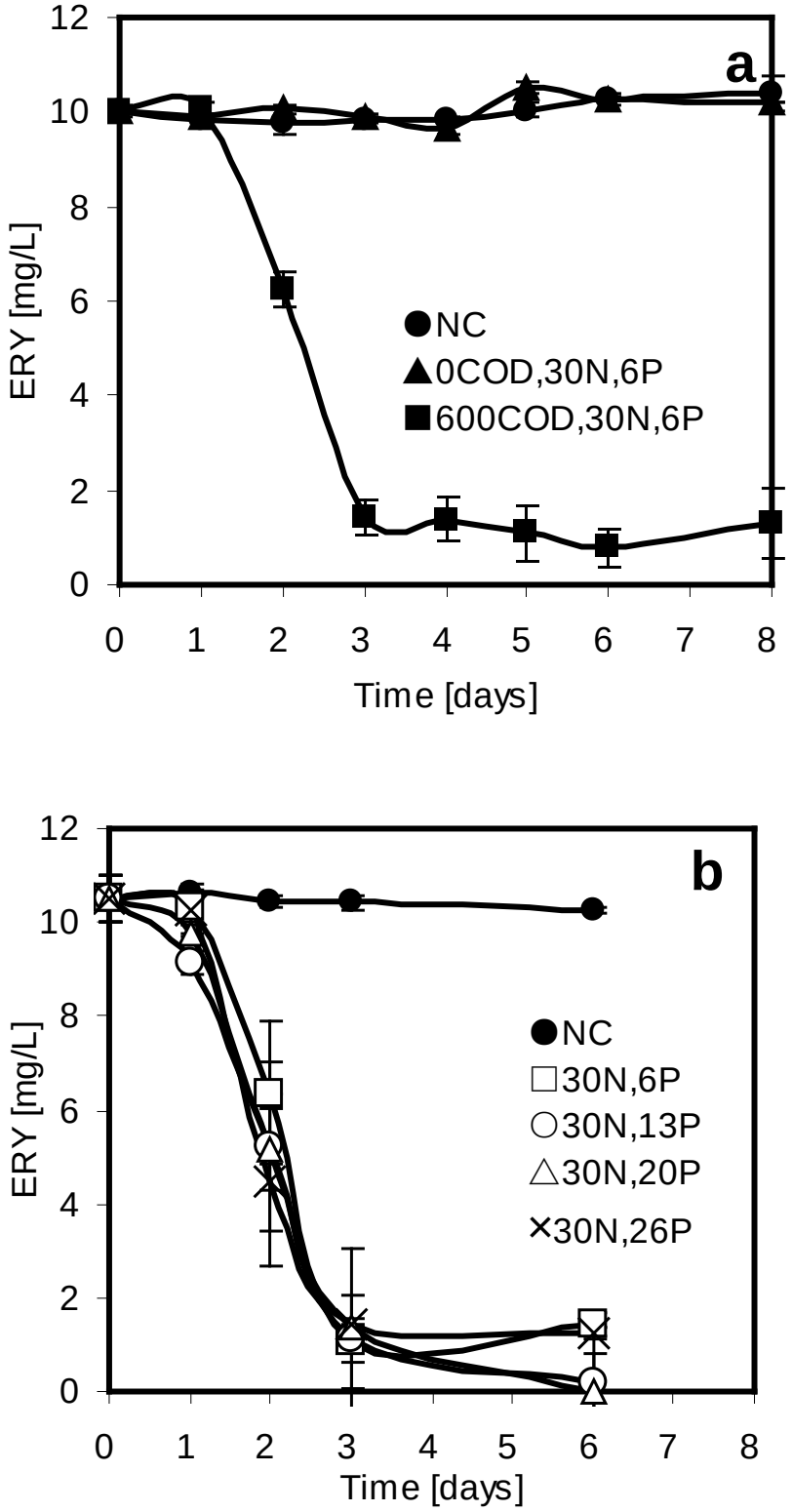
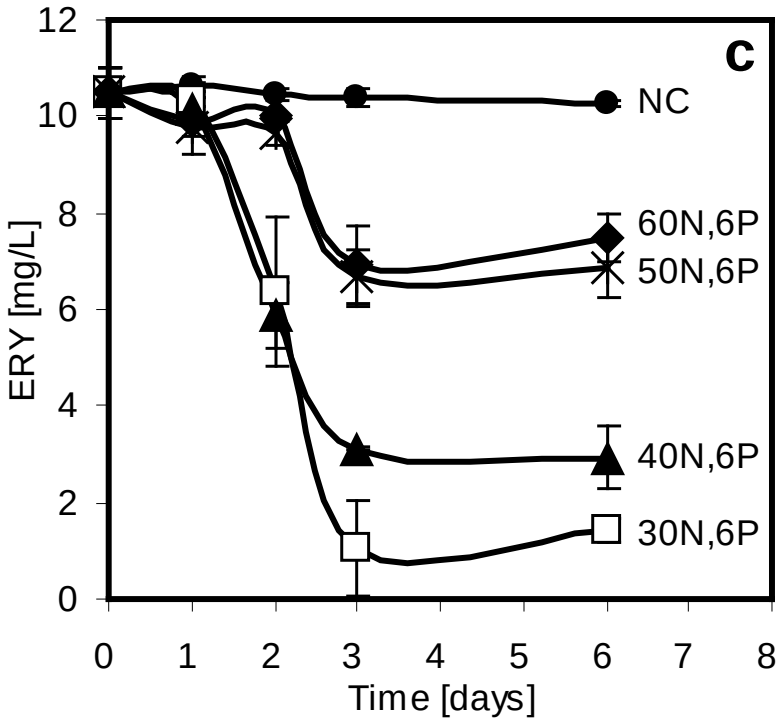


Fig. 4.6 The effects of **a** — glucose, and **b** — phosphate on the biodegradation of ERY. The values represent the means ± standard deviations (n=3). NC means negative control.



Continued Fig. 4.6 The effects of **c** — ammonium on the biodegradation of ERY. The values represent the means ± standard deviations (n=3). NC means negative control.

4.4.4 Shift of microbial communities due to ERY biodegradation

T-RFLP and clone library were employed to find out the shift of microbial communities due to ERY biodegradation.

Change of community structure due to ERY biodegradation

The DNAs extracted from batch samples that ERY (10 mg/L) was completely biodegraded by inocula from R2 (ERY) were applied to T-RFLP analysis. The T-RFLP results showed that microbial community changed during ERY biodegradation process (Fig. 4.7). At the end of ERY degradation, T-RFLP distinguished three dominant T-RFs with the sizes of 206 base pairs (bp), 492 bp and 121 bp digested by the enzymes *Hha*I, *Msp*I, and *Rsa*I, respectively.

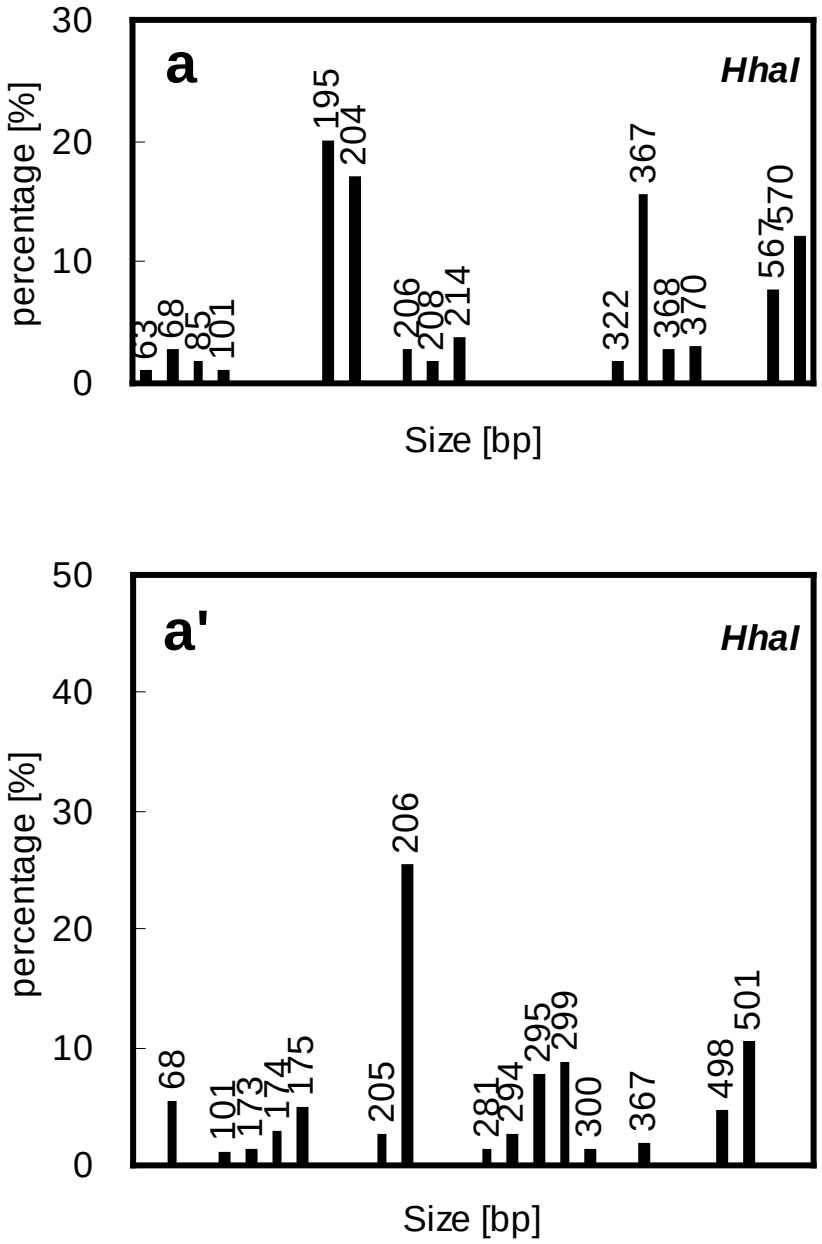
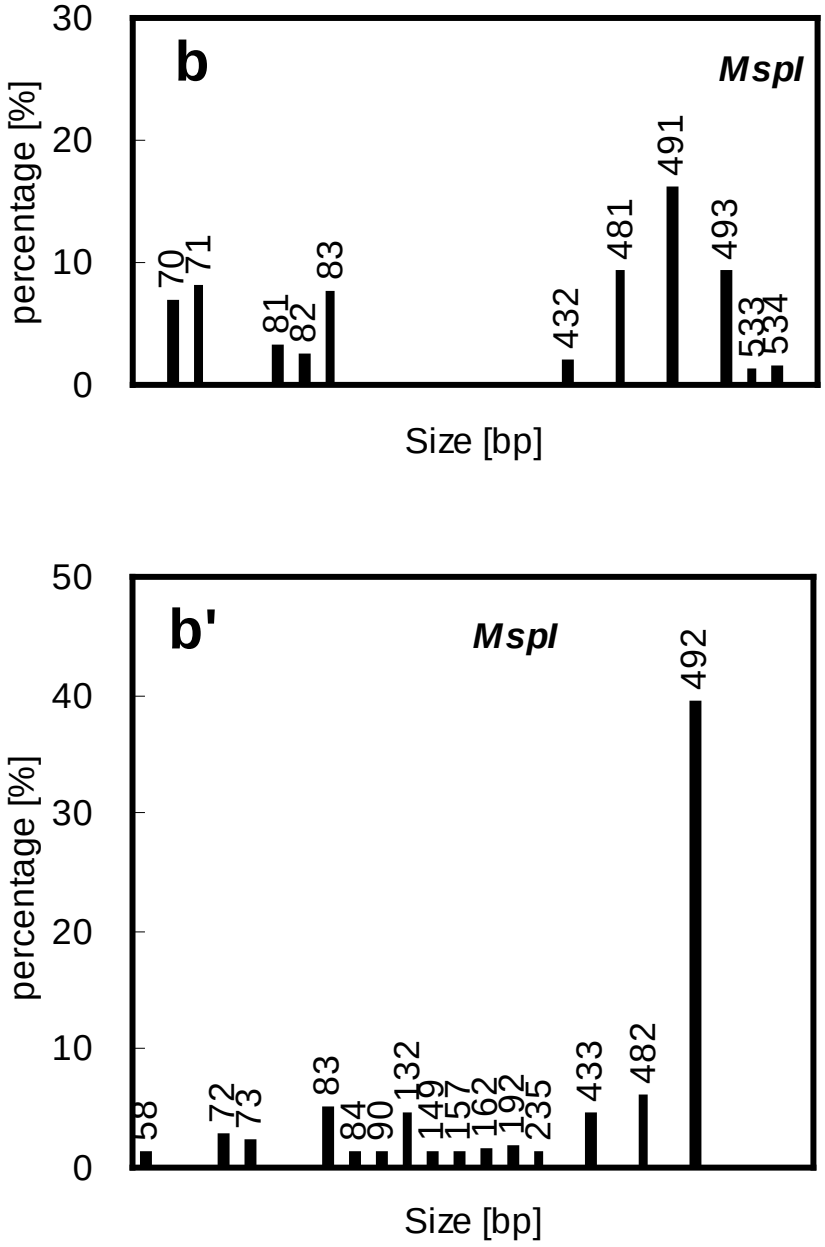
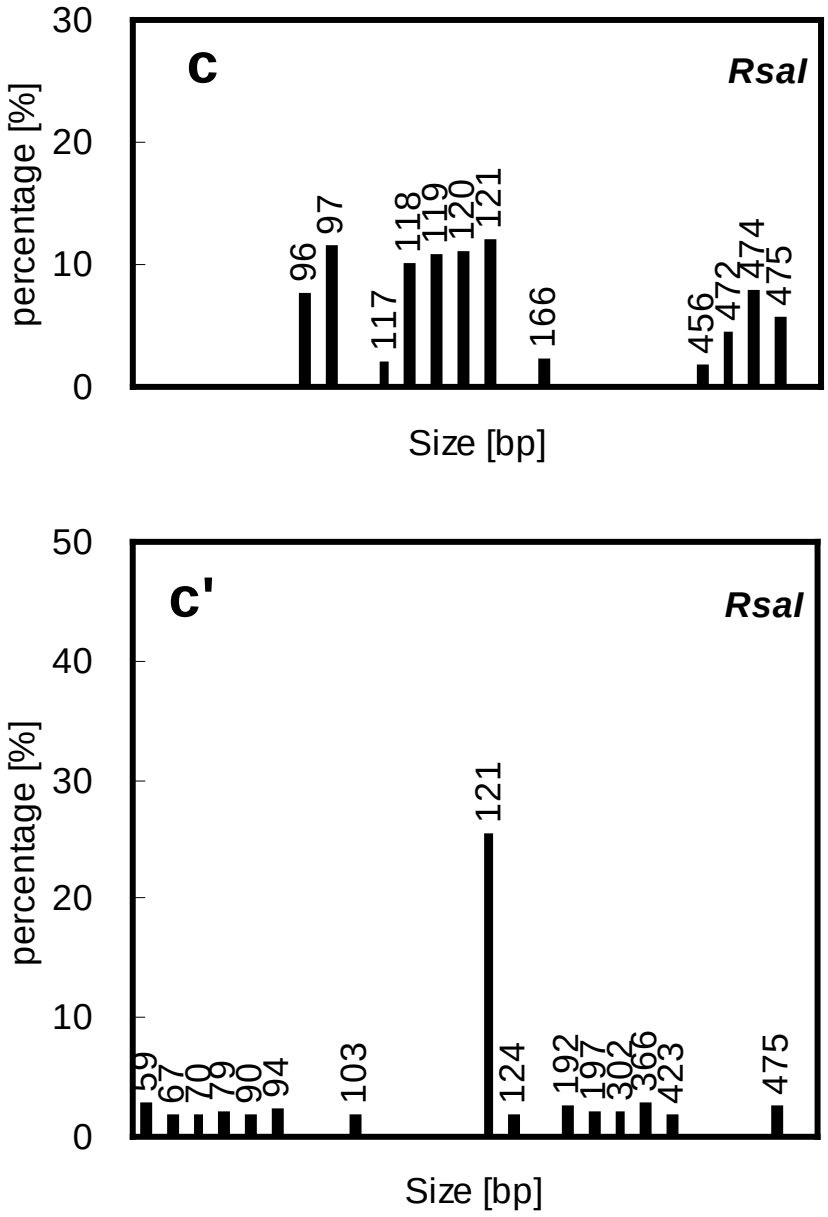


Fig. 4.7 T-RFLP results for the samples in the degradation batches of R2 (ERY). **a, b** and **c** — the sample on day 0 before ERY degradation; **a', b'** and **c'** — the sample on the day that ERY was completely degraded. Peaks less than 1% were not shown.



Continued Fig. 4.7 T-RFLP results for the samples in the degradation batches of R2 (ERY). **a, b** and **c** — the sample on day 0 before ERY degradation; **a', b'** and **c'** — the sample on the day that ERY was completely degraded. Peaks less than 1% were not shown.



Continued Fig. 4.7 T-RFLP results for the samples in the degradation batches of R2 (ERY). **a, b** and **c** — the sample on day 0 before ERY degradation; **a', b'** and **c'** — the sample on the day that ERY was completely degraded. Peaks less than 1% were not shown.

Identification of the predominant species by clone library

To identify the species represented by the predominant T-RFs, a 16S rRNA clone library was established with the same genomic DNA as that in the T-RFLP. Restriction analysis of amplified ribosomal DNA (by *Msp*I and *Hha*I) generated two

dominant patterns (16 and 10 clones from 72 clones, respectively). Sequence results of the two patterns revealed that they were reversely complementary. Therefore, 36% of the clones represented one dominant species. The sequence results also showed that the dominant species possess 98% similarity of the 16S rRNA gene sequence of *Zoogloea* (DQ413151.1), a well-known producer of extracellular polymeric substances (EPS) that is vital in maintaining activated sludge floc. Consequently, bacteria in floc or biofilm increase antibiotic resistance 10 to 1,000 folds comparing to their planktonic formation (Anderson and O'Toole, 2008). Also, the dominant species exhibit the same T-RFs as those dominant T-RFs in the T-RFLP results based on the analysis of sequence by BioEdit assembly software.

4.5 Discussion

The presence of antibiotics and the continuous input of resistant bacteria and genes are considered as two main factors for the amplification of resistance (Kummerer, 2009b). Here in the absence of continuous resistant microorganisms and genes input via using synthetic wastewater, proliferation of esterase gene *ere*A as well as esterificaiton of ERY to 6 products was observed with microbes exposed to ERY (100 µg/L) for over one year, indicating that long-term exposure to ERY can induce resistance development in microbes. Therefore, the causal relationship of antibiotics at concentrations as low as found in the environment with expansion of resistance genes is strongly supported in this study by experimental data rather than by hypothesis in previous studies (Kummerer, 2009b). This discovery is inconsistent with other findings that ERY (up to 100 µg/L) is too low to sustain antibiotic resistance and continuous introduction of resistance genes may be more important for resistance expansion in the sewage (Ohlsen et al., 2003; Ohlsen et al., 1998). The

difference may be due to an over-simplified microbial community (two kinds of bacteria, one donor and one recipient for resistance genes) being operated over short time (10 days) in those studies, which may underestimate the transfer frequency and sustain of resistance genes in the ecosystem.

The ERY derivatives (e.g., ERY-H_2O) can be very persistent (Kummerer, 2009a), which makes complete mineralization of ERY difficult (Alexy et al., 2004; Gartiser et al., 2007b). The intermediates during antibiotics degradation was usually reported to possess negligible antibacterial activity due to that bacteria can directly destruct antibiotic functional groups or modify the macrolide antibiotics to gain resistance (Schlunzen et al., 2001; Wright, 2005). However, they may also induce microbial resistance to the parent drugs (Fan et al., 2009; Majer, 1981). Current study on ERY-H_2O further proved the above resistance development principle, although the developed resistance gene *ere*A in this study cannot induce enzyme immediately to degrade ERY. Similar as ERY-H_2O, other biodegradation products of ERY may also show no antibiotic activity due to the lost of several antibiotic functional –OH groups but may still induce microbial resistance to ERY (Schlunzen et al., 2001). Further study is needed to find out whether the degradation products of other antibiotics can still induce resistance to their parent drugs as ERY-H_2O does to ERY (Fan et al., 2009; Majer, 1981).

Antibiotics or their structure closely related derivatives at sub-inhibitory concentrations may act as signals to trigger specific response in micro-ecosystem to resistant antibiotics (Fajardo and Martinez, 2008). Erythromycin at sub-inhibitory concentrations has been reported to activate expression of specific gene encoding for polysaccharide (one of important components of EPS) intercellular adhesion in *Staphylococcus* (Rachid et al., 2000). Thus, ERY and *Zoogloea* may follow similar

interactions as shown in the above study. Erythromycin may trigger the Gram-negative *Zoogloea* to become dominant and to form protective biofilms, since the presence of intrinsic efflux resistance genes in Gram-negative bacteria can make them more impermeable and less sensitive to ERY (Pechere, 2001). Bacteria containing *ere*A gene are speculated to be located at the outer edges of *Zoogloea* formed biofilm to eliminate antibiotics from harming the inner microbes. In turn, both microbes containing *ere*A gene and biofilm cells work together to provide sufficient antibiotic resistance in the microbial communities. Moreover, long-term treatment of bacteria with lower antibiotic concentrations has been discovered to result in higher resistant capability of biofilm populations of *E. coli* than planktonic bacteria (Harrison et al., 2005). Further study is needed to clarify gene-based biofilm resistance to antibiotics at low concentrations (e.g., in WWTPs). Notably, full-scale WWTPs may show more frequent resistance gene development and transfer than in this study, which could be caused by (1) wastewater properties–complex wastewater versus synthetic wastewater; (2) diversity of microbial communities–complex versus enriched microbial consortia; (3) frequent resistant bacteria and genes input versus free of these two sources (Szczepanowski et al., 2009; Zhang et al., 2009). Also, since ERY is sensitive to pH, it may be dehydrated into ERY-H_2O in wastewater collection pipelines before entering WWTPs. Consequently, ERY does not have a chance to be esterified in WWTPs, but ERY-H_2O becomes prevalent there as reported previously (Le-Minh et al., 2010).

Higher concentrations of ammonium (NH_4^+–N>40 mg/L) were found to inhibit ERY biodegradation for more than 30% (Fig. 4.6), which may be explained by the toxicity of free ammonia (when NH_4^+–N–N =60 mg/L, pH=8.1, T=27°C, NH_3–N =4.6 mg/L) to most microorganisms. Considering NH_4^+–N of 60 mg/L in the

synthetic wastewater was diluted to a final concentration of 30 mg/L in the influent of R2 (ERY of 100 µg/L, a dilution factor of 2) (Fan et al., 2009), this concentration of ammonia did not inhibit the ERY biodegradation. However, in the sewage treatment plants (STPs) receiving antibiotics discharged from both hospitals and households, ammonium concentrations fluctuate during the day (could be > 40 mg/L) and dilution factors are process-dependent (usually < 2), which is more likely to inhibit the biodegradation of ERY. Moreover, the even higher concentrations of ammonium in separate sewage system and in pharmaceutical wastewater will definitely make ERY biodegradation more difficult to occur. The untreated ERY, in turn, will inhibit ammonium oxidization (Alexy et al., 2004; Nimenya et al., 1999). Awareness is needed to optimize STPs to cope with this problem.

4.6 Conclusions

In conclusion, both ERY-H_2O (50 µg/L) and ERY (100 µg/L) can encourage development of ERY esterase gene *ere*A under conditions of free input of resistant bacteria and genes. Dehydrated erythromycin acclimated microbes cannot esterify ERY as ERY acclimated ones do, which may be due to the less proliferated *ere*A gene. This study suggests that the presence of ERY and ERY-H_2O at concentrations as low as found in the environment can enhance establishment of antibiotic resistance, and will provide important information to substantiate correlation between resistance proliferation and antibiotics at sub-inhibitory concentrations.

Chapter 5

Loss of Bacterial Diversity and Enrichment of Betaproteobacteria in Microbial Consortia of SBRs Exposed to Trace ERY and ERY-H_2O

5.1 Abstract

The increasing use of antibiotics may contribute to the selection of antibiotic resistant bacteria in micro-ecosystems. This has led us to study long-term effects of antibiotics ERY (100 µg/L) and its derivative ERY-H_2O (50 µg/L) on the SBR microbial communities. Culture-independent analyses on 16S rRNA genes, including T-RFLP, denaturing gradient gel electrophoresis (DGGE) and microarrays (PhyloChip), were applied in this study. Results revealed that both ERY and ERY-H_2O had similar inhibitory and selective spectrum to significantly alter the phylogenetic structures of the SBR microbial communities. The Gram-positive Actinobacteria and Gram-negative Proteobacteria were inhibited in terms of both diversity and abundance. The selected bacteria were enriched in abundance, which belonged to the TM7 phylum, as well as the β-Proteobacteria subphylum within the Rhodocyclaceae family (within the genera of *Azonexus*, *Dechloromonas*, *Thauera*, and *zoogloea*) and the *Nitrosomonas* genus. The enriched *zoogloea* are capable of forming biofilm to resist antibiotics, and the nitrate-reduction *Azonexus*, *Dechloromonas* and *Thauera*, as well as the ammonium-oxidization *Nitrosomonas* are able to remove the toxic nitrogenous substances accumulated in the biofilm. With phylogenetic analysis on uncultured samples, our results suggested that low levels of ERY or ERY-H_2O can affect micro-communities by the inhibition of sensitive bacteria and the enrichment of biofilm antibiotic resistant bacteria.

5.2 Introduction

The sub-inhibitory concentrations of antibiotics in the environment are suspected to account for the rapid development and spreading of resistant pathogens, which greatly imperil the therapeutic usage of antibiotics. The antibiotics in the

environment are mainly released via anthropogenic activities, the massive production and the improper utilization of antibiotics (e.g., in the treatment of virus-caused diseases and in the promotion of animal growth) (Aminov, 2009; Davies and Davies, 2010). However, both efforts to discover the new antibiotics and to control the usage of antibiotics have failed to effectively cope with the proliferation of antibiotic resistant microorganisms. This is due to the lack of knowledge on the natural roles of antibiotics in the environment. Different from higher to lethal concentrations of antibiotics that act as a stress to inhibit or kill microorganisms, sub-inhibitory concentrations of antibiotics in the environment are recently recognized to play the signaling and regulatory roles in micro-ecosystems (Davies et al., 2006; Linares et al., 2006; Martinez, 2008; Yim et al., 2006). For example, low-dose antibiotics have been found to definitely induce antibiotic resistance genes as the genotypic response of the opportunistic pathogen *Pseudomonas aerugenosa*, and to increase/decrease alginate production and biofilm volume as their phenotypic response (Aminov, 2009). Most of this updated knowledge on the natural roles of antibiotics was based on the investigations of pure isolates. Few studies have been conducted on the microbial communities exposed to the low concentrations of antibiotics in the environment, where the regulation impact of antibiotics are expected to be more complex due to the higher density and diversity of microorganisms.

As the main collection pools of antibiotics and antibiotic resistant bacteria, WWTPs are posing increasing threat to human beings because of the wide usage of the recycled wastewater (e.g., as portable and non-portable water sources) (Le-Minh et al., 2010). The antibiotics erythromycin (ERY) and its derivative ERY-H_2O are with the lowest removal rate in WWTPs (Rosal et al., 2010). Therefore, ERY and ERY-H_2O are most frequently detected in downstream water bodies (e.g., surface

water, ground water and untreated drinking water sources) (Focazio et al., 2008). Many types of ERY resistance genes (ribosomal methylase genes *erm* (A, B, C, E, F, T, V, and X), efflux genes *mef* (A, E, and I) and *msr*A, esterase genes *ere*A/B, and macrolide-2'-phosphotransferase gene *mph*A) have been detected in wastewater and activated sludge of WWTPs (Szczepanowski et al., 2009; Zhang et al., 2009). And the proliferation of *ere*A gene has been reported as the result of low concentrations of ERY or ERY-H_2O in the lab-scale SBRs (Fan and He, 2011). As phenotypic response to sub-inhibitory concentrations of ERY, isolate *Staphylococcus* have been reported to activate expression of specific gene encoding polysaccharide (one of important components of biofilm extracellular polymeric substances (EPS) matrix) for intercellular adhesion (Rachid et al., 2000). Biofilm populations of *Escherichia coli* have been discovered to possess higher resistant capability than planktonic bacteria when treated with lower concentrations of antibiotics for longer time (Harrison et al., 2005). However, the phenotypic response of microbial communities to low-dose antibiotics (e.g. ERY) in WWTPs has rarely been conducted. This is due to the lack of antibiotics-uncontaminated WWTPs as negative controls and the disturbance of antibiotic resistant bacteria brought by wastewater. In addition, there is a limit of technologies that can comprehensively target the massive un-culturable microorganisms in the environment (e.g., WWTPs). All these reasons may result in the difficulties to discover the less pronounced effects of antibiotics at lower concentrations (Ding and He, 2010).

Since the majority of microorganisms in natural environments, such as WWTPs, are not culturable (Amann et al., 1995), uncultured-methods are developed to detect, characterize and quantify microbes in natural systems. These uncultured-methods are usually based on molecular approaches, classifying heterogeneous

nucleic acids via the universal 16S rRNA genes (DeSantis et al., 2007). The tools used to categorize 16S rRNA genes include clone libraries to identify the bacterial species present, as well as the rapid and high-throughput methods of T-RFLP and denaturing gradient gel electrophoresis (DGGE) to qualitatively describe the spatial and temporal changes in microbial communities. Different from above tools that only profile the dominant organisms of a complex bacterial population and have to accompany sequencing to characterize taxonomic nomenclature of each group, a higher-density and more throughput oligonucleotide microarray (PhyloChip) was designed to target more taxa by vastly increasing the total number of probes and has been successfully applied in different environmental samples (DeSantis et al., 2007; DeSantis et al., 2005; Hazen et al., 2010).

The aim of this study is to identify phylogenetic structure change of SBR microbial consortia due to being acclimated to ERY (100 µg/L) or ERY-H_2O (50 µg/L) for over one-year running with synthetic wastewater free from resistant bacteria and resistance genes input. The 16S rRNA genes based uncultured methods, including T-RFLP, DGGE and PhyloChip microarray, were used to statistically evaluate shift of microbial communities and further to identify enriched and inhibited microbial taxa. The result will help to enlarge the knowledge about effects of low dose antibiotics to regulate microbes in WWTPs.

5.3 Materials and methods

5.3.1 DNA extraction, PCR, and T-RFLP

The startup, operation methods and performance data of the three SBRs were described in chapter 3. The genomic DNA of mixed liquid from the SBRs were extracted with the method described in chapter 3. According to the methods

described in chapter 4, each DNA pool extracted from triplicate sludge samples was duplicated applied to PCR for the amplification of the 16S rRNA genes with eubacterial primers 8F-cy5 (Zhou et al., 1995) and 1392R (Lane et al., 1985), the amplified 16S rRNA genes were digested with 5 U of restriction endonucleases *Hha*I (NEB, USA) (He et al., 2003) for following T-RFs determination, and then the individual T-RFs were normalized as a percentage of the total peak area.

5.3.2 PhyloChip

The high-density phylogenetic 16S rRNA gene microarrays (PhyloChip) employed in this study contain 1,440 distinguishable prokaryotic operational taxonomic units (OTUs) identified by 35,000 probes, which were designed according to the approach described previously (DeSantis et al., 2003). Each DNA pool extracted from triplicate sludge samples was tested on triplicate PhyloChips. The archaeal and bacterial 16S rRNA gene were amplified using PCR with primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') for bacteria and 4Fa (5'-TCCGGTTGATCCTGCCRG-3') and 1492R for archaea according to the previous method (Hazen et al., 2010). PhyloChip processing (Brodie et al., 2006; Hazen et al., 2010), scanning, OTU scoring, and normalization were all performed as previously described (Brodie et al., 2006; DeSantis et al., 2007; Flanagan et al., 2007; Hazen et al., 2010) with some minor modification. A probe pair was considered positive when the difference in intensity between the perfect match (PM) and mismatch (MM) probes must be at least 130 times the squared noise value (N). A taxon was considered to be present in the sample needs two criteria: (1) 90% or more of its assigned probe pairs for its corresponding probe set were positive (positive fraction,

≥0.90), and (2) positive fractions of this taxon tested in triplicate PhyloChip were all ≥0.90. After probe intensities were array-normalized by scaling to the internal standards, a significantly linear correlation was established between the $\log_2$(concentrations) of each internal standard gene and the corresponding intensity (average correlation coefficient r = 0.0029, and average intercept = -1.4501). Therefore, a change in intensity of 400 units is approximate to a 2.23-fold change in gene copy number.

5.3.3 PCR–DGGE

PCR-DGGE analyses were carried out for universal bacterial 16S rRNA gene amplified with primer pair 8F-GC (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGAGAGTTTGATCCTGGCTCAG-3') (Muyzer et al., 1993; Reysenbach et al., 1994) and 518R (5'-ATTACCGCGGCTGGCTGG-3') (Muyzer et al., 1993). PCR amplified fragments were electrophoresed on an 8% polyacrylamide gel with a 30–70% urea–formamide gradient for 16 h at 120V and 60°C.

Selected DGGE band fragments were excised with sterile razors, washed with sterile deionized water, and then placed in 50 µL of DNA elution buffer (QIAGEN GmbH, Germany) overnight. The eluted DNA was used in PCR reaction with primers without GC-clamps, 8F and 518R. The PCR products were purified with PCR purification kit (QIAGEN GmbH, Germany) and subsequently sequenced on an ABI 3100 Sequencer (Applied Biosystems). Sequences were analyzed with BioEdit assembly software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and BLAST with similar sequences in GenBank (http://www.ncbi.nlm.nih.gov/). Phylogenetic

tree of 16S rRNA gene sequenced from DGGE bands were constructed with the neighbor-jointing method ordination of ρ-distance by software MEGA 4.

5.3.4 Statistical analysis

Nonmetric Multidimensional Scaling

Nonmetric multidimensional scaling (NMDS) plots facilitate the exploration of similarities and dissimilarities through visualization of data sets. To obtain a NMDS plot, a similarity matrix derived from the data sets of 16S rRNA gene sequences, including (A) the PhyloChip intensities after normalized with whole chip intensities and (B) the percentages of T-RFs, were first generated based on Bray-Curtis distance with or without square-root transformation, respectively. The matrix then undergoes a 100-iterations NMDS analysis. Nonmetric multidimensional scaling plot analyses were subsequently performed with the software Primer 5 (http://www.primer-e.com/).

Indentifying dynamic subfamilies

An OTU consists of a group of one or more 16S RNA sequences with typically 97 to 100% sequence homology, while a subfamily consists of a group of OTUs with typically no less than 94% sequence homology.

When summarizing PhyloChip results to subfamily, the taxon with a probe set producing the highest mean intensity within the subfamily was used. To identify the most dynamic 16S rRNA gene amplicons, hierarchical clustering was performed on a prefiltered subfamilies list containing only the 100 most variable subfamilies (based on standard deviation). This was performed with the software Cluster 3.0. Both samples and subfamilies were clustered with Pearson correlation as the distance

metric and unweighted pair group method with arithmetic mean (UPGMA) as the linkage method. The cluster result was visualized with the software TreeView.

Indentifying variable OTUs and subfamilies

In case of losing information of OTUs with non-highest intensity within a subfamily, statistical analysis was firstly performed on all taxa and then the results were summarized to subfamily. Significantly inhibited OTUs in R1 (ERY-H_2O of 50ug/L) or R2 (ERY of 100μg/L) were defined as those present in R3 sample, achieving a probability values $p<0.05$ with Student's *t*-test upon $\log_2$(concentration), and an decrease in mean intensity compared to R3 (control) of >400 units and >30%. Significantly enriched OTUs in R1 and R2 were defined similarly, except an increase in mean intensity. Significantly distinct OTUs in R1 (ERY-H_2O of 50ug/L) relative to R2 (ERY of 100μg/L) were defined as those present in R1 sample, achieving a probability values $p<0.05$ with Student's *t*-test upon $\log_2$(concentration), and a difference in mean intensity compared to R2 of >400 units and >30%. When summarizing PhyloChip results to subfamily, the taxon with a probe set producing the highest percent difference in mean intensity within the subfamily was used.

5.4 Results

5.4.1 NMDS analysis of bacterial population shifts

Archaeal and bacterial 16S rRNA genes were amplified and analysed on PhyloChip microarray. PhyloChip detected only two archaeal OTUs that belong to Euryarchaeota Phylum, indicating that archaea can be ignored in these three SBR systems. Analysis of bacterial 16S rRNA genes by PhyloChip microarray and TRFLP suggested that ERY (100 μg/L in R2) and ERY-H_2O (50 μg/L in R1) significantly

altered the bacterial community composition and structure (Fig.5.1). Ordination of bacterial 16S rRNA gene composition revealed three distinct clusters of samples from R1, R2 and R3, respectively. No any other physical or chemical factors were different between these groups except the presence/absence of ERY or ERY-H_2O, indicating that bacteria were responding directly to the presence of ERY or ERY-H_2O.

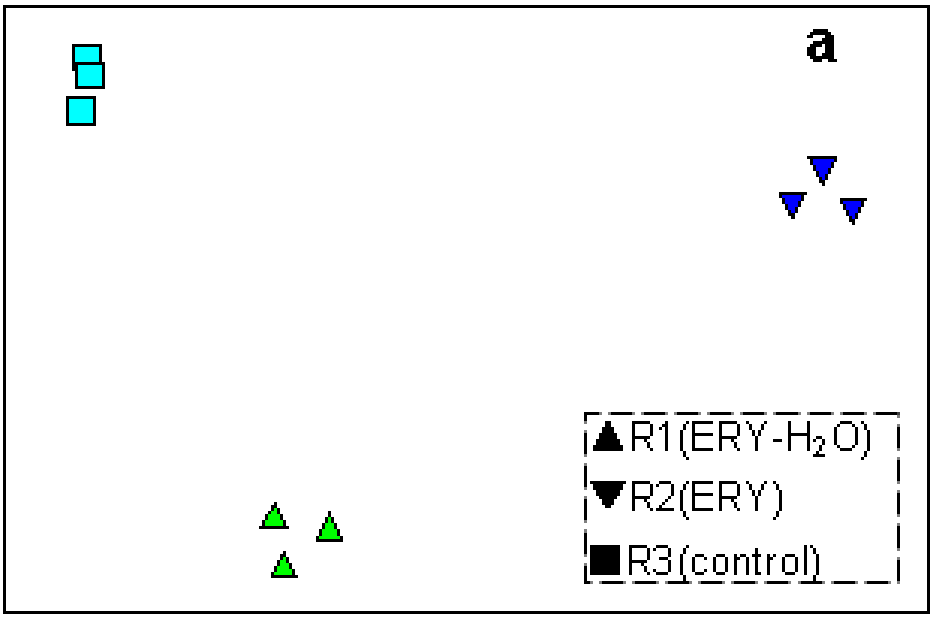
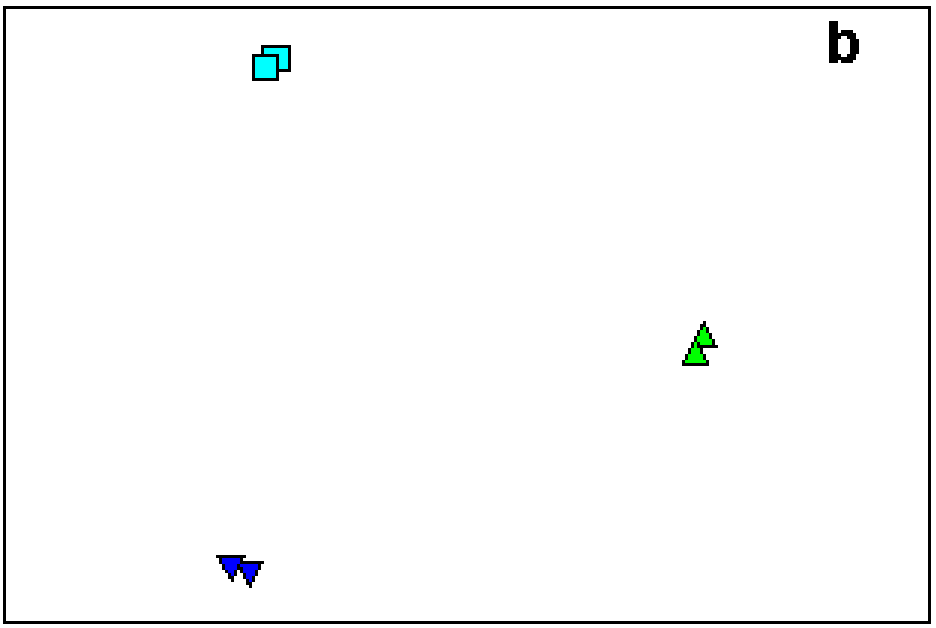


Fig. 5.1 Microbial community analysis of R1 (ERY-H_2O), R2 (ERY) and R3 (control) samples. Differences in composition of 16S rRNA gene sequences (**a**) measured by PhyloChip were analyzed using nonmentric multidimensional scaling (NMDS) ordination of Bray-Curtis distance (stress = 0.01 and 0, respectively). Communities of R1, R2 and R3 were clustered well apart from each other and can be significantly differentiated



Continued Fig. 5.1 Microbial community analysis of R1 (ERY-H_2O), R2 (ERY) and R3 (control) samples. Differences in composition of 16S rRNA gene sequences (**b**) based on terminal restriction fragments (T-RFs) were analyzed using nonmentric multidimensional scaling (NMDS) ordination of Bray-Curtis distance (stress = 0.01 and 0, respectively). Communities of R1, R2 and R3 were clustered well apart from each other and can be significantly differentiated

5.4.2 Bacterial richness identified by Phylochip analysis

PhyloChip analysis revealed that 825, 699 and 920 distinct bacterial taxa belonging to 37 phyla were present in R1 (ERY-H_2O), R2 (ERY) and R3 (control) respectively, indicating that both ERY-H_2O and ERY had a pronounced effect on bacterial community diversity, with taxonomic richness falling (Fig. 5.2 and 5.3). .

Taxonomic richness of R1 and R2 decreased significantly in bacteria classified as Gram-positive Actinobacteria (55, 28 and 62 taxa detected in R1, R2 and R3) and Gram-negative Proteobacteria (449, 354 and 509 taxa detected in R1, R2 and R3), which is consistent with that ERY has a broad antimicrobial spectrum of affecting both Gram-positive and Gram-negative bacteria, as well as suggests that ERY-H_2O had the similar inhibitory spectrum as ERY (Amin et al., 2006). Among Proteobacteria, only the richness of Betaproteobacteria decreased significantly in

richness due to ERY-H_2O, while the richness of Proteobacteria within all α, β, γ, δ, and ε subphyla decreased due to ERY, indicating that inhibitory spectrum of ERY-H_2O was not as broad as ERY.

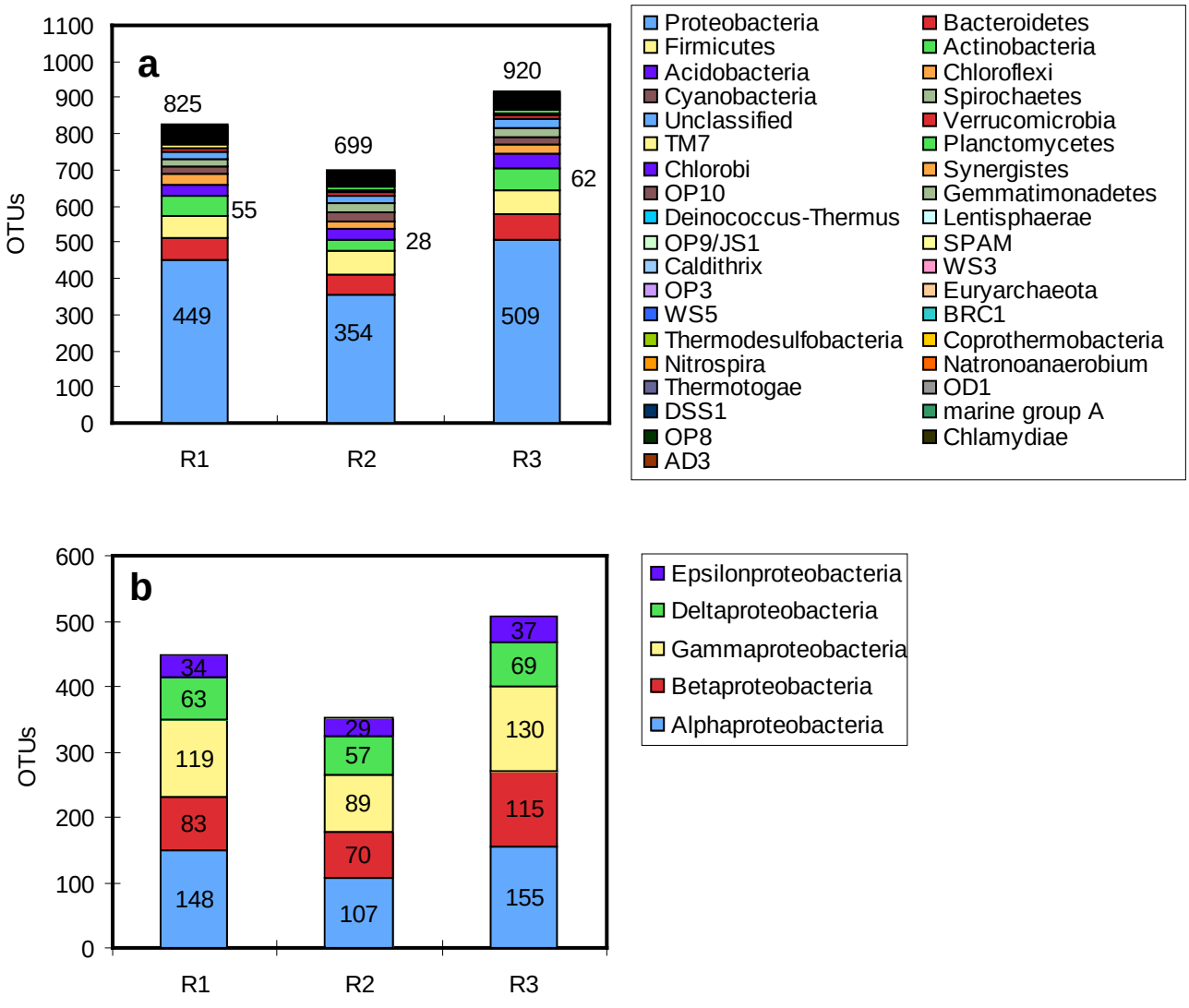


Fig. 5.2 Bacterial richness detected in R1 (ERY-H_2O), R2 (ERY) and R3 (control) samples. Using PhyloChip analysis, **(a)** a total of 825, 699 and 920 OTUs in 37 bacterial phyla were detected in samples R1, R2 and R3, respectively. Taxonomic richness of bacteria in phylum Proteobacteria significantly decreased in R1 (50 µg/L of ERY-H_2O) and R2 (100 µg/L of ERY) compared to R3 (control). And richness of bacteria in phylum Actinobacteria also significantly decreased in R2 compared to R3. **(b)** Bacteria richness in all subphyla of Proteobacteria decreased in R1 and R2 compared to R3

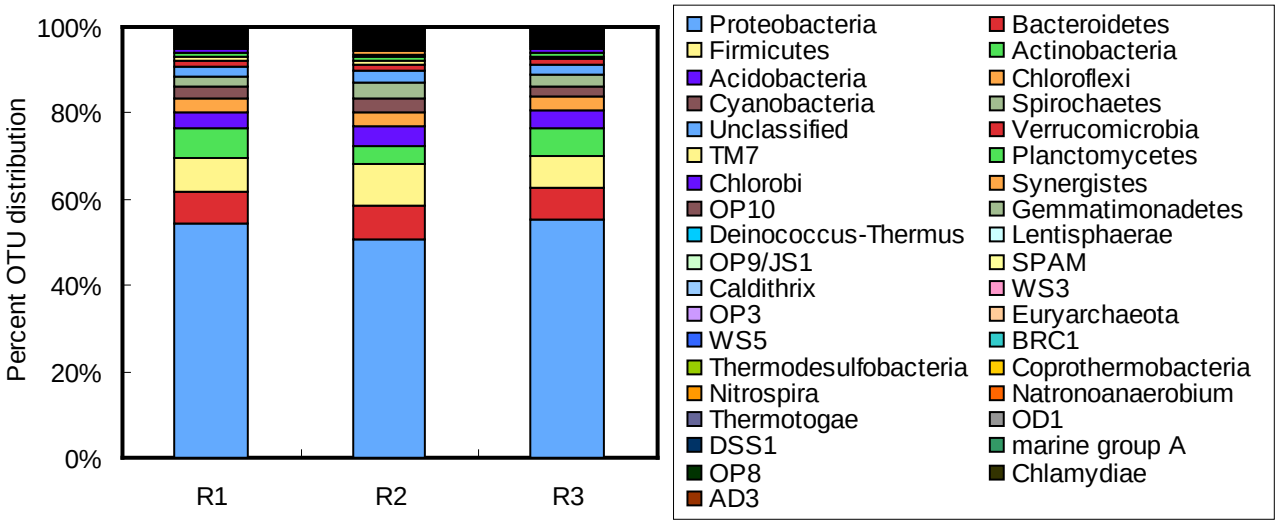


Fig. 5.3 Phylum-level distribution of bacterial OTUs from R1 (ERY-H_2O), R2 (ERY) and R3 (control) samples. Percent of taxonomic richness of Proteobacteria and Actinobacteria bacteria was significantly decreased in R2 (100 µg/L of ERY) compared to R3 (control)

5.4.3 Most dynamic subfamilies identified by Phylochip analysis

An OUT producing the highest mean intensity within the subfamily was used as the representative for summarizing PhyloChip results to subfamily. Dynamic subfamilies were detected by confining the analysis to the 100 subfamilies exhibiting the greatest standard deviation between the SBRs sampled. Hierarchical cluster analysis allowed detection of correlations between samples and also between subfamilies (Fig. 5.4). Array analysis indicated that R2 (ERY) sample communities formed a separate cluster from those sampled from R1 (ERY-H_2O) and R3 (control) with the latter two arising from a common node. Overall, three groups of dynamic subfamilies were detected by cluster analysis (Fig. 5.4; see Table 5.1 of subfamilies). All the three cluster groups comprised subfamilies which were of lower abundance in R1 (ERY-H_2O) and R2 (ERY) communities comparing with R3 (control), indicating that inhibition was the main effect of ERY and ERY-H_2O on bacteria.

Cluster group 1 was a large cluster containing sequences from 57 bacterial subfamilies whose amplicons were of the lowest abundance in R1 (ERY-H_2O) community relative to R2 (ERY) and R3 (control). This group contained mostly Proteobacteria with some Acidobacteria, Actinobacteria, Bacteroidetes, and a few members of the Caldithrix, Chlorobi, Chloroflexi, Coprothermobacteria, Cyanobacteria, Firmicutes, Gemmatimonadetes, SPAM and TM7 phyla. The Proteobacteria which responded in this manner were mostly orders within α (Acetobacterales, Azospirillales, Caulobacterales, Ellin314/wr0007, Ellin329/Riz1046, Rhodobacterales, Rickettsiales, Sphingomonadales), γ (Chromatiales, GAO cluster, Legionellales, Methylococcales, Oceanospirillales, Thiotrichales, Xanthomonadales) and δ (Desulfobacterales, Desulfovibrionales, Myxococcales, Syntrophobacterales) subphyla. Two orders of Betaproteobacteria, Nitrosomonadales (capable of ammonium oxidizing) and Rhodocyclales (as nitrate reducer), and an Epsilonproteobacteria of the order Campylobacterales also responded in a similar way.

Cluster group 2 consisted of Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes, OP 10, OP9/JS1, Proteobacteria and Synergistes sequences from 34 bacterial subfamilies, whose abundance was of the lowest in R2 (ERY) community. This group contained the Acidobacteria within two classes, Acidobacteria-10 and Solibacteres. Bacteria of the Actinobacteria were classified as families (Cellulomonadaceae, Corynebacteriaceae, Kineosporiaceae, Micrococcaceae, and Unclassified Actinomycetales) within the order of Actinomycetales, many of which are pathogens for human. All three of the subfamilies from the Bacteroidetes were orders within Bacteroidales and Sphingobacteriales. And the subfamilies from the Chloroflexi were unclassified. In addition, members of the Gram-positive

Firmicutes were also detected within the families of Clostridiaceae, Lachnospiraceae and Peptostreptococcaceae, specifically the family of Clostridiaceae containing incredibly dangerous human pathogens *Clostridium.* This cluster also contained Proteobacteria within orders of α (Bradyrhizobiales, Rhizobiales, Sphingomonadales), γ (Legionellales) and δ (AMD clone group, Desulfobacterales) subphyla.

The final cluster group observed, group 3, comprised nine subfamilies which were of significantly lower abundance in R2 (ERY) bacteria than R1 (ERY-H_2O). Two of the subfamilies were from the Actinobacteria within the families of Actinomycetaceae and Micromonosporaceae, with both representative clones (AJ428402.1 and AJ277568.1) being known to cause disease in humans and plants. One of the subfamily was from the class of Nitrospira, with members of Gram-negtive nitrite-oxidising organism. The other nine members of cluster group 3 were Proteobacteria, with eight subfamilies from the orders of α (Azospirillales, Bradyrhizobiales, Rhizobiales) subphyla.

In summary, analysis of 100 most dynamic subfamilies indicated that inhibition, the main effect of ERY-H_2O and ERY on bacteria, mostly influenced on Actinobacteria and α, γ and δ subphyla of Proteobacteria, but less on β and ε Proteobacteria. In addition to Proteobacteria and Actinobacteria, Bacteroidetes and Chloroflexi were also affected by both ERY-H_2O and ERY. And Firmicutes, Nitrospira and OP10 were inhibited by ERY more significantly than ERY-H_2O.

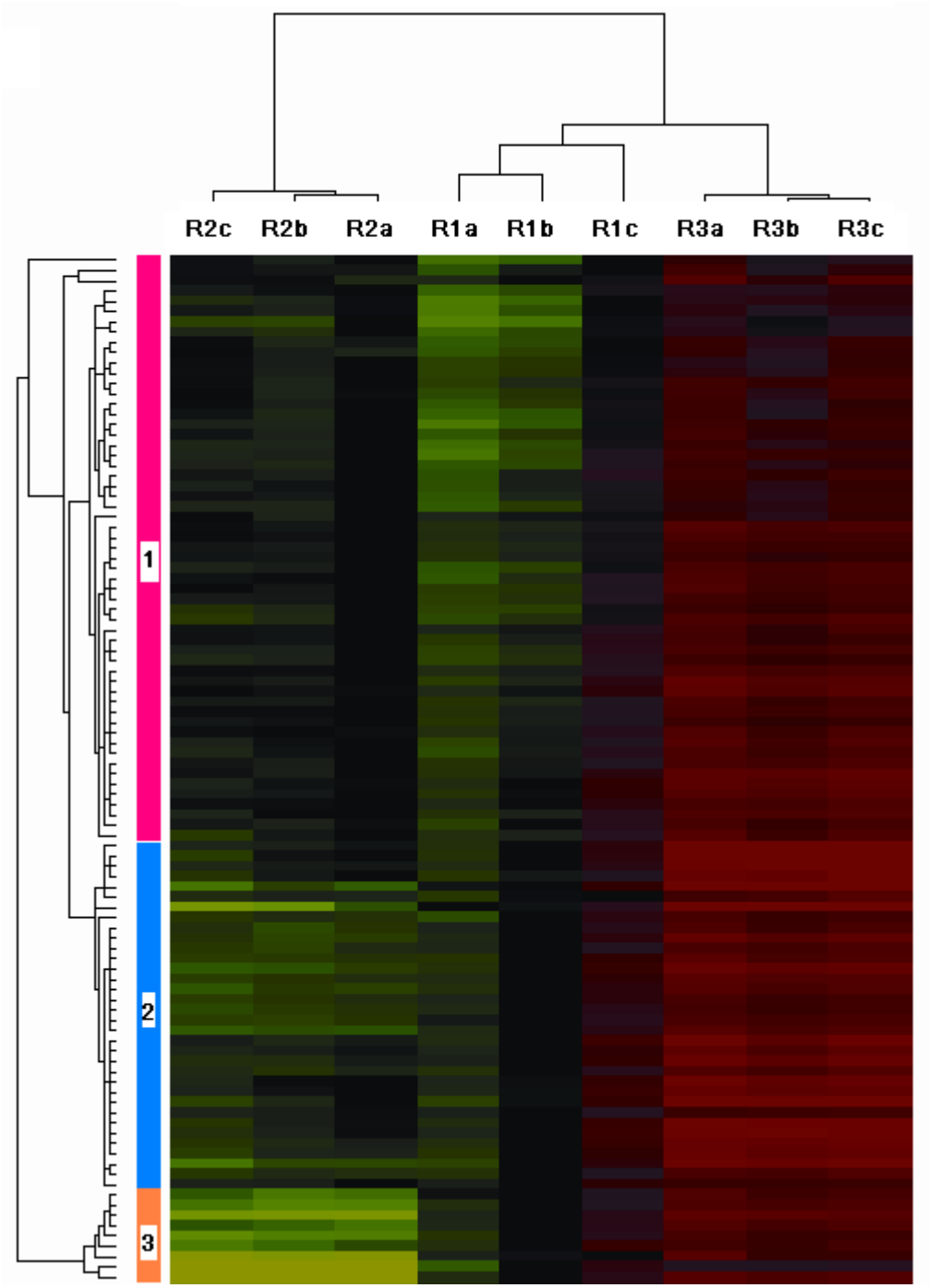


Fig. 5.4 Hierarchical cluster analysis showing the response of 100 most dynamic bacterial subfamilies (shown on *y* axis) exhibiting the highest standard deviation between R1 (ERY-H_2O), R2 (ERY) and R3 (control) samples (shown on *x* axis). The color gradient from green, black to red represents gene intensity (after log transformation and median centered within the subfamily) from negative, zero to positive. Three main response groups were detected (Table 5.1)

Table 5.1 One hundred most dynamic bacterial subfamilies

Group 1: Domain;Phylum;Class;Order;Family;Subfamily;OTU-ID

1. Bacteria;Proteobacteria;Betaproteobacteria;Rhodocyclales;Rhodocyclaceae;sf_1;7824
2. Bacteria;Cyanobacteria;Cyanobacteria;Chloroplasts;Chloroplasts;sf_5;5147
3. Bacteria;Proteobacteria;Alphaproteobacteria;Ellin329/Riz1046;Unclassified;sf_1;6945
4. Bacteria;Acidobacteria;Acidobacteria-6;Unclassified;Unclassified;sf_1;834
5. Bacteria;Chloroflexi;Anaerolineae;Chloroflexi-1a;Unclassified;sf_1;583
6. Bacteria;Proteobacteria;Gammaproteobacteria;Legionellales;Unclassified;sf_1;9418
7. Bacteria;Actinobacteria;BD2-10 group;Unclassified;Unclassified;sf_2;1652
8. Bacteria;TM7;TM7-3;Unclassified;Unclassified;sf_1;2917
9. Bacteria;Proteobacteria;Alphaproteobacteria;Acetobacterales;Unclassified;sf_1;7028
10. Bacteria;Proteobacteria;Alphaproteobacteria;Ellin314/wr0007;Unclassified;sf_1;7123
11. Bacteria;Proteobacteria;Alphaproteobacteria;Acetobacterales;Acetobacteraceae;sf_1;7529
12. Bacteria;Proteobacteria;Gammaproteobacteria;Xanthomonadales;Xanthomonadaceae;sf_3;8689
13. Bacteria;Proteobacteria;Alphaproteobacteria;Sphingomonadales;Unclassified;sf_1;6653

Group 1: Domain;Phylum;Class;Order;Family;Subfamily;OTU-ID

14. Bacteria;Proteobacteria;Alphaproteobacteria;Unclassified;Unclassified;sf_6;8780
15. Bacteria;Proteobacteria;Betaproteobacteria;Nitrosomonadales;Nitrosomonadaceae;sf_1;7789
16. Bacteria;Proteobacteria;Gammaproteobacteria;Legionellales;Coxiellaceae;sf_3;7893
17. Bacteria;Proteobacteria;Gammaproteobacteria;Chromatiales;Chromatiaceae;sf_1;9054
18. Bacteria;Proteobacteria;Unclassified;Unclassified;Unclassified;sf_21;8509
19. Bacteria;Proteobacteria;Deltaproteobacteria;Syntrophobacterales;Syntrophaceae;sf_3;9665
20. Bacteria;Proteobacteria;Deltaproteobacteria;Desulfobacterales;Desulfobulbaceae;sf_1;9739
21. Bacteria;Unclassified;Unclassified;Unclassified;Unclassified;sf_106;243
22. Bacteria;Firmicutes;Clostridia;Clostridiales;Peptococc/Acidaminococc;sf_11;992
23. Bacteria;Proteobacteria;Epsilonproteobacteria;Campylobacterales;Campylobacteraceae;sf_3;10447
24. Bacteria;Proteobacteria;Epsilonproteobacteria;Campylobacterales;Unclassified;sf_1;10427
25. Bacteria;SPAM;Unclassified;Unclassified;Unclassified;sf_1;738
26. Bacteria;Proteobacteria;Alphaproteobacteria;Sphingomonadales;Sphingomonadaceae;sf_1;6663

Group 1: Domain;Phylum;Class;Order;Family;Subfamily;OTU-ID

27. Bacteria;Bacteroidetes;Bacteroidetes;Bacteroidales;Unclassified;sf_15;5890
28. Bacteria;Bacteroidetes;Sphingobacteria;Sphingobacteriales;Crenotrichaceae;sf_11;6267
29. Bacteria;Bacteroidetes;Bacteroidetes;Bacteroidales;Porphyromonadaceae;sf_1;5454
30. Bacteria;Bacteroidetes;Sphingobacteria;Sphingobacteriales;Sphingobacteriaceae;sf_1;5913
31. Bacteria;Proteobacteria;Gammaproteobacteria;Methylococcales;Unclassified;sf_1;9182
32. Bacteria;Coprothermobacteria;Unclassified;Unclassified;Unclassified;sf_1;751
33. Bacteria;Acidobacteria;Acidobacteria-5;Unclassified;Unclassified;sf_1;523
34. Bacteria;Chloroflexi;Dehalococcoidetes;Unclassified;Unclassified;sf_1;2497
35. Bacteria;Actinobacteria;Actinobacteria;Bifidobacteriales;Bifidobacteriaceae;sf_1;1351
36. Bacteria;Proteobacteria;Alphaproteobacteria;Rickettsiales;Anaplasmataceae;sf_3;6648
37. Bacteria;Chlorobi;Unclassified;Unclassified;Unclassified;sf_9;6146
38. Bacteria;Bacteroidetes;Sphingobacteria;Sphingobacteriales;Flexibacteraceae;sf_19;5372
39. Bacteria;Acidobacteria;Acidobacteria;Acidobacteriales;Acidobacteriaceae;sf_14;6368

Group 1: Domain;Phylum;Class;Order;Family;Subfamily;OTU-ID

40. Bacteria;Unclassified;Unclassified;Unclassified;Unclassified;sf_160;6430
41. Bacteria;Bacteroidetes;Bacteroidetes;Bacteroidales;Bacteroidaceae;sf_12;5256
42. Bacteria;Proteobacteria;Alphaproteobacteria;Rhodobacterales;Unclassified;sf_5;7471
43. Bacteria;Actinobacteria;Actinobacteria;Acidimicrobiales;Acidimicrobiaceae;sf_1;1090
44. Bacteria;Proteobacteria;Gammaproteobacteria;Oceanospirillales;Alcanivoraceae;sf_1;8335
45. Bacteria;Proteobacteria;Alphaproteobacteria;Rickettsiales;Unclassified;sf_1;7156
46. Bacteria;Proteobacteria;Epsilonproteobacteria;Campylobacterales;Helicobacteraceae;sf_3;10448
47. Bacteria;Proteobacteria;Gammaproteobacteria;GAO cluster;Unclassified;sf_1;9008
48. Bacteria;Proteobacteria;Deltaproteobacteria;Desulfovibrionales;Desulfohalobiaceae;sf_1;9894
49. Bacteria;Gemmatimonadetes;Unclassified;Unclassified;Unclassified;sf_5;442
50. Bacteria;Proteobacteria;Alphaproteobacteria;Azospirillales;Unclassified;sf_1;7400
51. Bacteria;Proteobacteria;Deltaproteobacteria;Desulfobacterales;Desulfoarculaceae;sf_2;10227
52. Bacteria;Caldithrix;Unclassified;Caldithrales;Caldithraceae;sf_1;2384

Group 1: Domain;Phylum;Class;Order;Family;Subfamily;OTU-ID

53. Bacteria;Proteobacteria;Alphaproteobacteria;Caulobacterales;Caulobacteraceae;sf_1;6909
54. Bacteria;Unclassified;Unclassified;Unclassified;Unclassified;sf_156;4291
55. Bacteria;Proteobacteria;Gammaproteobacteria;Thiotrichales;Francisellaceae;sf_1;9554
56. Bacteria;Proteobacteria;Deltaproteobacteria;Syntrophobacterales;Syntrophobacteraceae;sf_1;9661
57. Bacteria;Proteobacteria;Deltaproteobacteria;Myxococcales;Polyangiaceae;sf_3;10353

Group 2: Domain;Phylum;Class;Order;Family;Subfamily;OTU-ID

1. Bacteria;Proteobacteria;Gammaproteobacteria;Legionellales;Legionellaceae;sf_1;8836
2. Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Cellulomonadaceae;sf_1;1748
3. Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Corynebacteriaceae;sf_1;1374
4. Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Unclassified;sf_3;1405
5. Bacteria;Bacteroidetes;Sphingobacteria;Sphingobacteriales;Unclassified;sf_8;5380
6. Bacteria;Bacteroidetes;Bacteroidetes;Bacteroidales;Bacteroidaceae;sf_6;5792

Group 2: Domain;Phylum;Class;Order;Family;Subfamily;OTU-ID

7. Bacteria;Bacteroidetes;Bacteroidetes;Bacteroidales;Prevotellaceae;sf_1;6011
8. Bacteria;Proteobacteria;Alphaproteobacteria;Bradyrhizobiales;Methylobacteriaceae;sf_1;7585
9. Bacteria;Acidobacteria;Solibacteres;Unclassified;Unclassified;sf_1;6367
10. Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Bradyrhizobiaceae;sf_1;7029
11. Bacteria;Proteobacteria;Alphaproteobacteria;Sphingomonadales;Sphingomonadaceae;sf_15;7035
12. Bacteria;OP10;Unclassified;Unclassified;Unclassified;sf_4;484
13. Bacteria;Chloroflexi;Chloroflexi-3;Roseiflexales;Unclassified;sf_5;119
14. Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Unclassified;sf_1;6899
15. Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Rhizobiaceae;sf_1;7051
16. Bacteria;Acidobacteria;Acidobacteria-10;Unclassified;Unclassified;sf_1;516
17. Bacteria;Firmicutes;Clostridia;Clostridiales;Clostridiaceae;sf_12;4272
18. Bacteria;Chloroflexi;Unclassified;Unclassified;Unclassified;sf_12;2523
19. Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Bartonellaceae;sf_1;7384

Group 2: Domain;Phylum;Class;Order;Family;Subfamily;OTU-ID

20. Bacteria;OP10;Unclassified;Unclassified;Unclassified;sf_5;9782
21. Bacteria;Synergistes;Unclassified;Unclassified;Unclassified;sf_3;353
22. Bacteria;Chloroflexi;Unclassified;Unclassified;Unclassified;sf_1;2534
23. Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;sf_5;4525
24. Bacteria;OP10;CH21 cluster;Unclassified;Unclassified;sf_1;514
25. Bacteria;OP9/JS1;OP9;Unclassified;Unclassified;sf_1;969
26. Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Kineosporiaceae;sf_1;1961
27. Bacteria;Chloroflexi;Anaerolineae;Unclassified;Unclassified;sf_9;375
28. Bacteria;Proteobacteria;Deltaproteobacteria;AMD clone group;Unclassified;sf_1;3084
29. Bacteria;Proteobacteria;Deltaproteobacteria;Desulfobacterales;Nitrospinaceae;sf_2;594
30. Bacteria;Proteobacteria;Deltaproteobacteria;Desulfobacterales;Unclassified;sf_3;9813
31. Bacteria;Firmicutes;Clostridia;Clostridiales;Unclassified;sf_17;4307
32. Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Micrococcaceae;sf_1;1266

Group 2: Domain;Phylum;Class;Order;Family;Subfamily;OTU-ID

33. Bacteria;Firmicutes;Clostridia;Clostridiales;Peptostreptococcaceae;sf_5;2913

34. Bacteria;Firmicutes;Clostridia;Unclassified;Unclassified;sf_7;4216

Group 3: Domain;Phylum;Class;Order;Family;Subfamily;OTU-ID

1. Bacteria;Nitrospira;Nitrospira;Nitrospirales;Nitrospiraceae;sf_1;860

2. Bacteria;Proteobacteria;Alphaproteobacteria;Azospirillales;Magnetospirillaceae;sf_1;6922

3. Bacteria;Proteobacteria;Alphaproteobacteria;Bradyrhizobiales;Bradyrhizobiaceae;sf_1;6799

4. Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Beijerinck/Rhodoplan/Methylocyst;sf_1;7591

5. Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Hyphomicrobiaceae;sf_1;7392

6. Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Actinomycetaceae;sf_1;1684

7. Bacteria;Proteobacteria;Unclassified;Unclassified;Unclassified;sf_28;10091

8. Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Micromonosporaceae;sf_1;1159

9. Bacteria;Proteobacteria;Alphaproteobacteria;Bradyrhizobiales;Beijerinck/Rhodoplan/Methylocyst;sf_3;6651

5.4.4 Variable subfamilies identified by Phylochip analysis

Different from 100 most dynamic subfamilies, whose PhyloChip results were firstly summarized to subfamily followed by statistical analysis, significantly variable subfamilies were analyzed on all taxa and then the results were summarized to subfamily in the way that the taxon with a probe set producing the highest percent difference in mean intensity within the subfamily was used.

Microarray analysis did not discover significantly enriched subfamily based on the filter conditions used in the statistical analysis. Comparing communities of R1 (ERY-H_2O) and R2 (ERY) with R3, most of significantly inhibited subfamilies in R1 (18 of 23 subfamilies) were also inhibited severely in R2 (Fig. 5.5, Table 5.2 and 5.3), indicating that ERY-H_2O (50 µg/L in R1) had the similar inhibitory spectrum as ERY (100 µg/L in R2). The subfamilies inhibited by both ERY-H_2O and ERY were mostly classified as α, γ and δ subphyla of Proteobacteria and the phyla of Actinobacteria, with some from the Bacteroidetes, Chloroflexi. The subfamilies from the Acidobacteria, Chlorobi, Firmicutes, Nitrospira and OP10 were only inhibited by ERY in R2. One subfamily within the Verrucomicrobia was only inhibited significantly by ERY-H_2O in R1, which was not included in the 100 most dynamic subfamilies.

The subfamilies significantly distinct between R1 and R2 were mostly within the subfamilies inhibited by both ERY-H_2O in R1 and ERY in R2, with relatively higher concentration of amplicons in R1 compared to R2, except the Verrucomicrobia with lower concentrations in R1 (Fig. 5.6 and Table 5.4). The results of distinct subfamilies between R1 and R2 suggested that the inhibitory effects of ERY-H_2O (50 µg/L in R1) on bacteria was not as significant as ERY, although having the similar inhibitory spectrum.

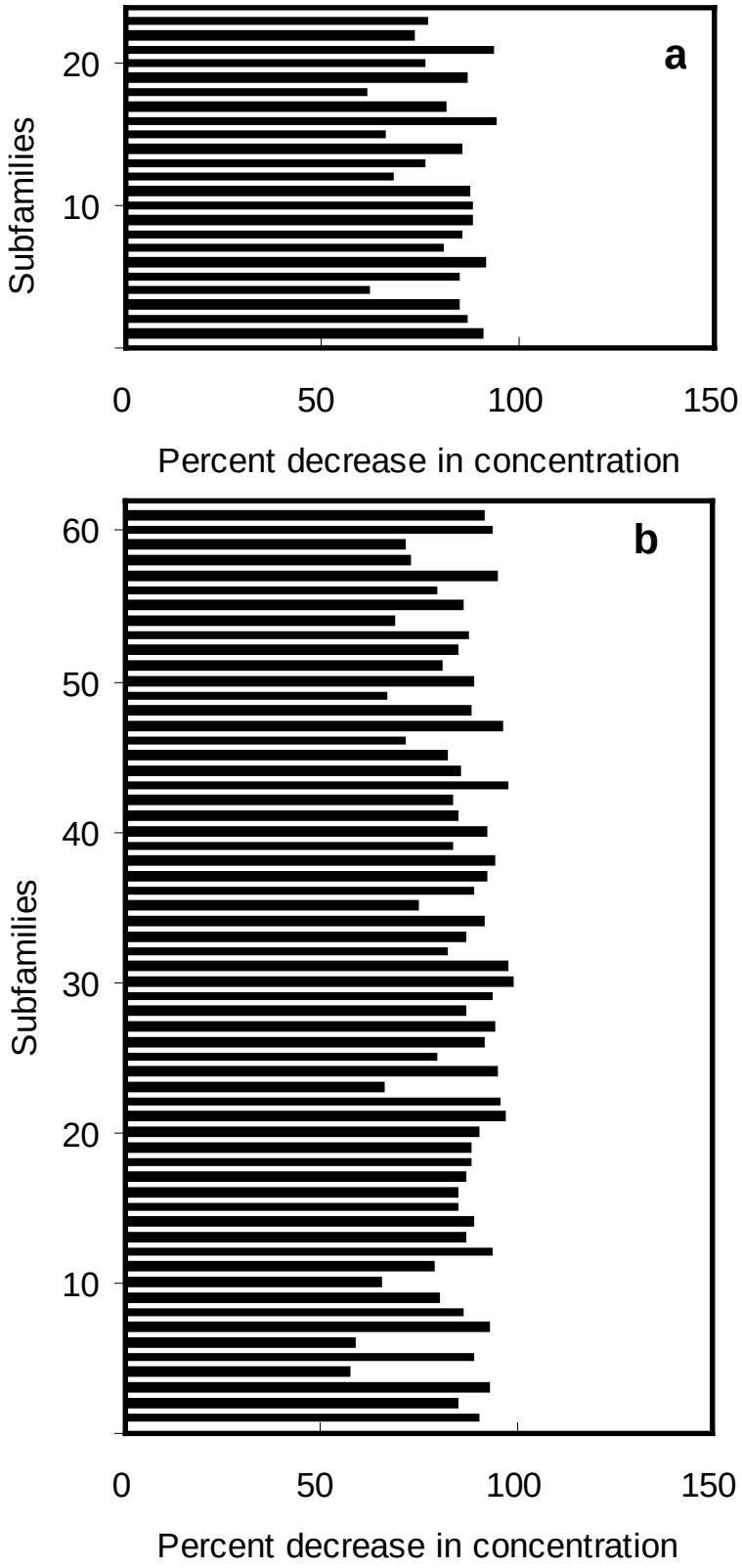


Fig. 5.5 Bacterial subfamilies inhibited by (a) 50 µg/L of ERY-H_2O in R1 and (b) 100 µg/L of ERY in R2. Differences in estimated 16S rRNA gene concentration are shown as percent of R3 (control) concentration for a representative OTU in each of the subfamilies that were significantly inhibited in R1 (Table 5.2, 23 representative OTUs) or R2 (Table 5.3, 61 representative OTUs) samples

Table 5.2 Bacterial subfamilies significantly inhibited by 50 µg/L of ERY-H_2O in R1

	Phylum	Class	Order	Family	Representative sequence	Concentration decreased (%)
1	Actinobacteria	Actinobacteria	Actinomycetales	Cellulomonadaceae	Y18378.1 Beutenbergia cavernosa str. DSM 12333	91
2	Actinobacteria	Actinobacteria	Actinomycetales	Corynebacteriaceae		87
3	Actinobacteria	Actinobacteria	Actinomycetales	Kineosporiaceae	X77958.1 Kineococcus aurantiacus str. IFO 15268	84
4	Actinobacteria	Actinobacteria	Actinomycetales	Kribbella	AY253862.1 Kribbella solani str. DSA1	62
5	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	AF134179.1 Arthrobacter psychrolactophilus	85
6	Actinobacteria	Actinobacteria	Actinomycetales	Unclassified	AJ626896.1 Jonesia quinghaiensis str. DSM 15701	92
7	Bacteroidetes	Bacteroidetes	Bacteroidales	Prevotellaceae	AB185583.1 rumen clone F24-B03	80
8	Bacteroidetes	Bacteroidetes	Bacteroidales	Unclassified	AJ534685.1 ground water deep-well injection disposal site radioactive wastes Tomsk-7 clone S15A-MN91	85
9	Bacteroidetes	Sphingobacteria	Sphingobacteriales	Crenotrichaceae	AJ290025.1 Austria: Lake Gossenkoellesee clone GKS2-106 GKS2-106	88
10	Bacteroidetes	Sphingobacteria	Sphingobacteriales	Unclassified	AJ007870.1 cytophagales clone LD1	88
11	Chloroflexi	Anaerolineae	Unclassified	Unclassified	AY218649.1 penguin droppings sediments clone	87

	Phylum	Class	Order	Family	Representative sequence	Concentration decreased (%)
					KD4-96	
12	Proteobacteria	Alphaproteobacteria	Bradyrhizobiales	Bradyrhizobiaceae	AB087719.1 Rhodopseudomonas rhenobacensis str. Klemme Rb	68
13	Proteobacteria	Alphaproteobacteria	Bradyrhizobiales	Unclassified	AF288309.1 Bosea massiliensis str. 63287	76
14	Proteobacteria	Alphaproteobacteria	Rhizobiales	Unclassified		85
15	Proteobacteria	Alphaproteobacteria	Unclassified	Unclassified	D88520.1 Stappia aggregata str. IAM12614	66
16	Proteobacteria	Betaproteobacteria	Rhodocyclales	Rhodocyclaceae	X74913.1 Zoogloea ramigera str. ATCC 19544 (T)	94
17	Proteobacteria	Deltaproteobacteria	AMD clone group	Unclassified	AF523883.1 coal effluent wetland clone RCP185	82
18	Proteobacteria	Deltaproteobacteria	Desulfobacterales	Desulfobulbaceae	AF099059.1 Psychrophilic sulfate-reducing isolate str. LSv23 bacterium	61
19	Proteobacteria	Deltaproteobacteria	Desulfovibrionales	Desulfovibrionaceae	AF228127.2 Desulfovibrio sp. str. Ac5.2	87
20	Proteobacteria	Gammaproteobacteria	Chromatiales	Chromatiaceae	AJ010297.2 Thiorhodovibrio sibirica	76
21	Proteobacteria	Gammaproteobacteria	Legionellales	Legionellaceae	CR628336.1 Legionella pneumophila str. Paris	94
22	Proteobacteria	Gammaproteobacteria	Methylococcales	Methylococcaceae		74
23	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobia subdivision 3	AF523995.1 coal effluent wetland clone FW4	76

Table 5.3 Bacterial subfamilies significantly inhibited by 100 µg/L of ERY in R2

	Phylum	Class	Order	Family	Representative sequence	Concentration decreased (%)
1	Acidobacteria	Acidobacteria	Acidobacteriales	Acidobacteriaceae	AY234728.1 Solibacter usitatus Ellin6076	90
2	Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetaceae	AJ428402.1 Varibaculum cambriense str. CCUG 44998	85
3	Actinobacteria	Actinobacteria	Actinomycetales	Cellulomonadaceae	Y18378.1 Beutenbergia cavernosa str. DSM 12333	93
4	Actinobacteria	Actinobacteria	Actinomycetales	Corynebacteriaceae	X84446.1 Corynebacterium xerosis str. DSM 20743	57
5	Actinobacteria	Actinobacteria	Actinomycetales	Kineosporiaceae	X77958.1 Kineococcus aurantiacus str. IFO 15268	89
6	Actinobacteria	Actinobacteria	Actinomycetales	Kribbella	AY253862.1 Kribbella solani str. DSA1	59
7	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	AF134179.1 Arthrobacter psychrolactophilus	92
8	Actinobacteria	Actinobacteria	Actinomycetales	Micromonosporaceae	AJ277568.1 Actinoplanes durhamensis str. IMSNU 22124T	86
9	Actinobacteria	Actinobacteria	Actinomycetales	Mycobacteriaceae	X52925.1 Mycobacterium terrae str. ATCC 15755	80
10	Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	AJ315953.1 Propionibacterium propionicum str. DSM 43307T	65
11	Actinobacteria	Actinobacteria	Actinomycetales	Sporichthyaceae	AY250883.1 lichen-dominated Antarctic cryptoendolithic community clone FBP417	79

	Phylum	Class	Order	Family	Representative sequence	Concentration decreased (%)
12	Actinobacteria	Actinobacteria	Actinomycetales	Unclassified	AJ626896.1 Jonesia quinghaiensis str. DSM 15701	94
13	Actinobacteria	Actinobacteria	Rubrobacterales	Rubrobacteraceae		87
14	Bacteroidetes	Bacteroidetes	Bacteroidales	Prevotellaceae	AB185583.1 rumen clone F24-B03	89
15	Bacteroidetes	Bacteroidetes	Bacteroidales	Rikenellaceae	AJ534686.1 ground water deep-well injection disposal site radioactive wastes Tomsk-7 clone S15A-MN128	85
16	Bacteroidetes	Bacteroidetes	Bacteroidales	Unclassified	AJ534685.1 ground water deep-well injection disposal site radioactive wastes Tomsk-7 clone S15A-MN91	85
17	Bacteroidetes	Sphingobacteria	Sphingobacteriales	Crenotrichaceae		87
18	Bacteroidetes	Sphingobacteria	Sphingobacteriales	Unclassified	AJ007870.1 cytophagales clone LD1	88
19	Chlorobi	Chlorobia	Chlorobiales	Chlorobiaceae	Y08105.1 Chlorobium phaeovibrioides str. 2631	88
20	Chloroflexi	Anaerolineae	Unclassified	Unclassified	AY218649.1 penguin droppings sediments clone KD4-96	90
21	Chloroflexi	Chloroflexi-3	Roseiflexales	Unclassified	AB079645.1 Green non-sulfur isolate str. B1-5	97
22	Chloroflexi	Unclassified	Unclassified	Unclassified	AF507693.1 forest soil clone S085	96
23	Firmicutes	Clostridia	Clostridiales	Clostridiaceae		66
24	Firmicutes	Clostridia	Clostridiales	Unclassified		95

	Phylum	Class	Order	Family	Representative sequence	Concentration decreased (%)
25	Firmicutes	gut clone group	Unclassified	Unclassified	AB185532.1 rumen clone F23-C12	79
26	Nitrospira	Nitrospira	Nitrospirales	Nitrospiraceae	AF033558.1 nitrifying sludge clone A-11	92
27	OP10	CH21 cluster	Unclassified	Unclassified	AF368184.1 sludge clone SBRA136	94
28	OP10	Unclassified	Unclassified	Unclassified	AF524022.1 forested wetland clone FW68	87
29	Proteobacteria	Alphaproteobacteria	Azospirillales	Magnetospirillaceae	AY171615.1 Dechlorospirillum sp. str. SN1	94
30	Proteobacteria	Alphaproteobacteria	Bradyrhizobiales	Beijerinck/Rhodoplan /Methylocyst	AB119196.1 Beijerinckia indica	99
31	Proteobacteria	Alphaproteobacteria	Bradyrhizobiales	Bradyrhizobiaceae	AB099659.1 Oligotropha carboxidovorans str. S23	97
32	Proteobacteria	Alphaproteobacteria	Bradyrhizobiales	Hyphomicrobiaceae	Y14304.1 Hyphomicrobium aestuarii str. DSM 1564	82
33	Proteobacteria	Alphaproteobacteria	Bradyrhizobiales	Unclassified	AF288309.1 Bosea massiliensis str. 63287	87
34	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	AB021414.1 Brevundimonas vesicularis str. IAM 12105T	91
35	Proteobacteria	Alphaproteobacteria	Consistiales	Caedibacteraceae	AJ428412.1 periodontal pocket clone 10B6	75
36	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bartonellaceae	U87831.1 aortic heart valve patient with endocarditis clone v9	89
37	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinck/Rhodoplan		92

	Phylum	Class	Order	Family	Representative sequence	Concentration decreased (%)
				/Methylocyst		
38	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae		94
39	Proteobacteria	Alphaproteobacteria	Rhizobiales	Brucellaceae		84
40	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae		92
41	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	U50164.1 Mesorhizobium loti str. R8CS USDA 3467	85
42	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	AF025852.1 Rhizobium huautlense str. SO2	83
43	Proteobacteria	Alphaproteobacteria	Rhizobiales	Unclassified		98
44	Proteobacteria	Alphaproteobacteria	Rhizobiales	Unclassified	X74915.1 Shinella zoogloeoides str. ATCC 19623	85
45	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Hyphomonadaceae	AJ227809.2 Maricaulis indicus str. MCS26	82
46	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	AF234741.1 sludge clone A6	71
47	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Unclassified	AF502220.1 EBPR sludge lab scale clone HP1B78	96
48	Proteobacteria	Alphaproteobacteria	Unclassified	Unclassified	D88520.1 Stappia aggregata str. IAM12614	88
49	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	AF236009.1 isolate str. A1004	67
50	Proteobacteria	Deltaproteobacteria	AMD clone group	Unclassified	AF523883.1 coal effluent wetland clone RCP185	89
51	Proteobacteria	Deltaproteobacteria	Desulfobacterales	Desulfobacteraceae		81
52	Proteobacteria	Deltaproteobacteria	Desulfobacterales	Nitrospinaceae	AJ296568.1 uranium mining mill tailing clone GR-296.II.52 GR-296.I.52	85
53	Proteobacteria	Deltaproteobacteria	Desulfobacterales	Unclassified	AF420340.1 hydrothermal	88

	Phylum	Class	Order	Family	Representative sequence	Concentration decreased (%)
					sediment clone AF420340	
54	Proteobacteria	Deltaproteobacteria	Myxococcales	Polyangiaceae	AJ518792.1 uranium mining waste pile clone JG37-AG-33 proteobacterium	68
55	Proteobacteria	Deltaproteobacteria	Syntrophobacterales	Syntrophobacteraceae	AJ518800.1 uranium mining waste pile clone JG37-AG-128 proteobacterium	86
56	Proteobacteria	Deltaproteobacteria	Unclassified	Unclassified	AY177804.1 Antarctic sediment clone LH5_30	79
57	Proteobacteria	Gammaproteobacteria	Legionellales	Legionellaceae	CR628336.1 Legionella pneumophila str. Paris	95
58	Proteobacteria	Gammaproteobacteria	Methylococcales	Methylococcaceae		72
59	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	AJ293826.1 Arctic seawater isolate str. R7366	71
60	Proteobacteria	Unclassified	Unclassified	Unclassified		93
61	Unclassified	Unclassified	Unclassified	Unclassified		91

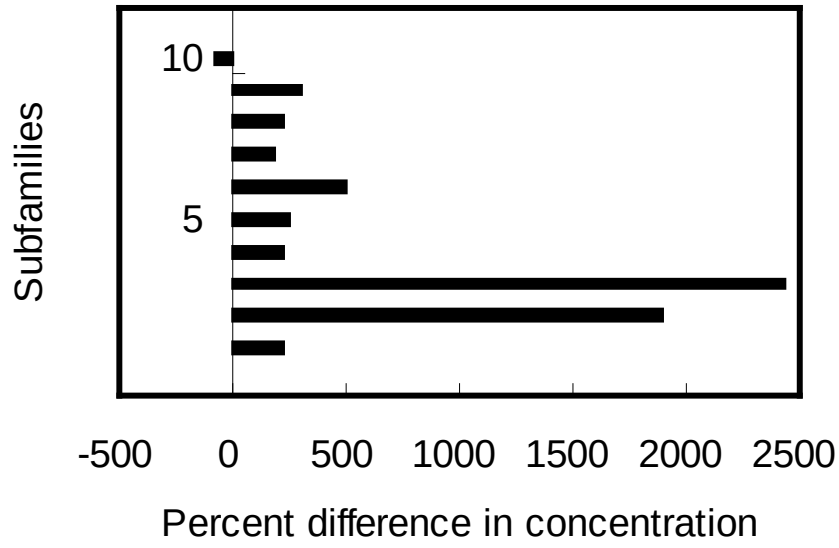


Fig. 5.6 Bacterial subfamilies differentiated between R1 (50 µg/L of ERY-H_2O) and R2 (100 µg/L of ERY). Differences in estimated 16S rRNA gene concentration are shown as percent of R2 concentration for a representative OTU in each of the subfamilies that were inhibited less significantly in R1 than in R2 samples (Table 5.4)

Table 5.4 Bacterial subfamilies differentiated between R1 (50 µg/L of ERY-H_2O) and R2 (100 µg/L of ERY)

	Phylum	Class	Order	Family	Representative sequence	Concentration difference (%)
1	Actinobacteria	Actinobacteria	Actinomycetales	Corynebacteriaceae	X84446.1 Corynebacterium xerosis str. DSM 20743	225
2	Proteobacteria	Alphaproteobacteria	Bradyrhizobiales	Beijerinck/Rhodoplan/ Methylocyst	AB119196.1 Beijerinckia indica	1897
3	Proteobacteria	Alphaproteobacteria	Bradyrhizobiales	Bradyrhizobiaceae	AB099659.1 Oligotropha carboxidovorans str. S23	2430
4	Proteobacteria	Alphaproteobacteria	Bradyrhizobiales	Unclassified	AB033757.1 Blastochloris sulfoviridis str. GN1	224
5	Proteobacteria	Alphaproteobacteria	Rhizobiales	Hyphomicrobiaceae		247
6	Proteobacteria	Alphaproteobacteria	Rhizobiales	Unclassified		505
7	Proteobacteria	Alphaproteobacteria	Unclassified	Unclassified	D88520.1 Stappia aggregata str. IAM12614	183
8	Proteobacteria	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	AF549390.1 Histophilus somni str. CCUG 12839	221
9	Proteobacteria	Unclassified	Unclassified	Unclassified		303
10	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Unclassified		-83

5.4.5 PCR-DGGE analysis of bacterial population shifts

In order to determine which bacterial group or taxon particular bands could be ascribed, specific DGGE band fragments that became intensified in the sample of R1 and R2 relative to R3 were further characterized by sequencing. In all, 8 different bands that became intensified in the sample of R1 (ERY-H_2O) and R2 (ERY) relative to R3 (control) were excised from the gel for further sequence (Fig. 5.7). The sequences obtained were compared with the NCBI database and phylogenetic tree was built, which were classified as the family of Rhodocyclaceae (capable of nitrate reduction), the genus of *Nitrosomonas* (able to oxidize ammonium) and the TM7 candidate division (Fig. 5.8). Bands 1–7 that intensified during exposure to ERY-H_2O belonged to the TM7 candidate division (band 5 and 6, 100% similarity) and the Gram-negative Betaproteobacteria within the families of Rhodocyclaceae (Band 1 and 2, 97% and 99% similar to *Zoogloea*; Band 3, 95% similar to *Thauera*; Band 7, 88% similar to *Azonexus*) and Notrosomonadaceae (Band 4, 97% similar to *Nitrosomonas*). Band 2, 4, 5 and 6 were also intensified during exposure to ERY, but Band 1 and 3 were not. Another Band 8 (97% similar to *Dechloromonas*) intensified due to ERY also belonged to the family of Rhodocyclaceae. These enriched bacteria are mostly related to bacterial biofilm or the so-called activated sludge floc, through which antibiotic stress was reduced (Anderson and O'Toole, 2008).

We also found that one of the subfamily inhibited by ERY-H_2O in R1 (Fig 5.5a, and Table 5.2) was from the family Rhodocyclaceae, with the representative sequence of *Zoogloea ramigera str*. (ATCC 19544, X74913.1). And another subfamily inhibited (Fig 5.4, and Table 5.1) was from the family of Notrosomonadaceae, with the representative clone of *Nitrosomonas*. Accordingly, even bacteria from the same genus (e.g., *Zoogloea* and *Nitrosomonas*) may be

impacted by ERY-H_2O (or ERY) in completely different ways, either inhibition or selection, which may depend on the absence/presence of antibiotic resistance genotypes or phonotypes.

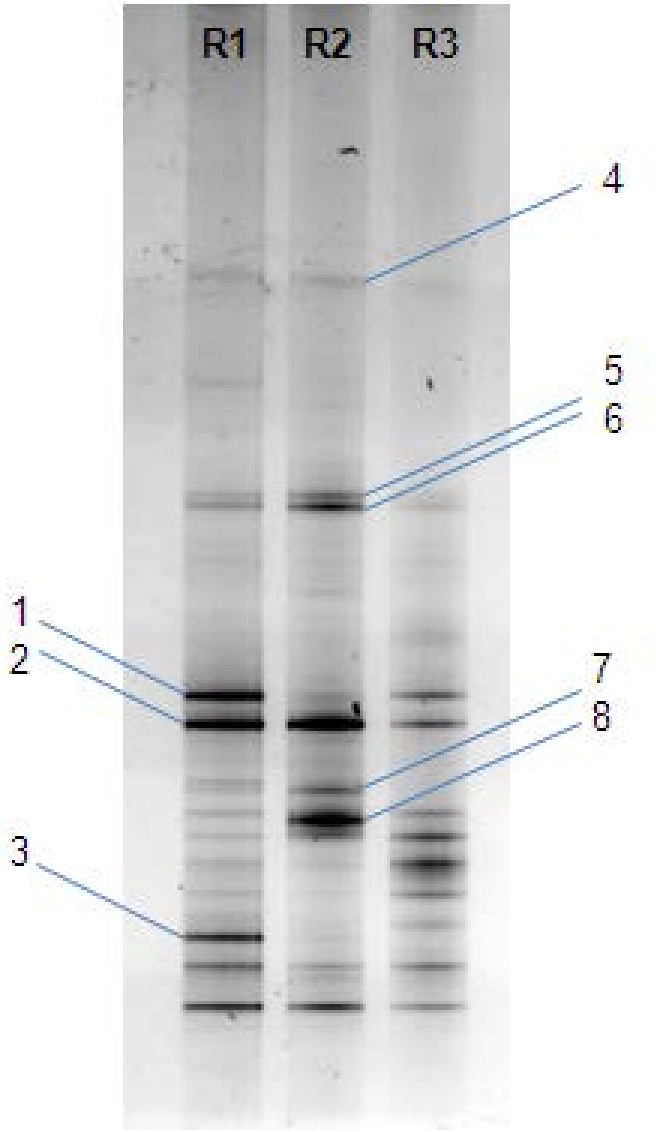


Fig. 5.7 DGGE profiles of microbes in R1 (ERY-H_2O), R2 (ERY) and R3 (control). Sequence blast results of DGGE bands intensified in R1 and R2 relative to R3 are: Band 1 and 2 Uncultured *Zoogloea*, Band 3 Uncultured *Thauera*, Band 4 Uncultured *Nitrosomonas*, Band 5 and 6 Uncultured TM7 bacterium, Band 7 Uncultured *Azonexus*, and Band 8 Uncultured *Dechloromonas*

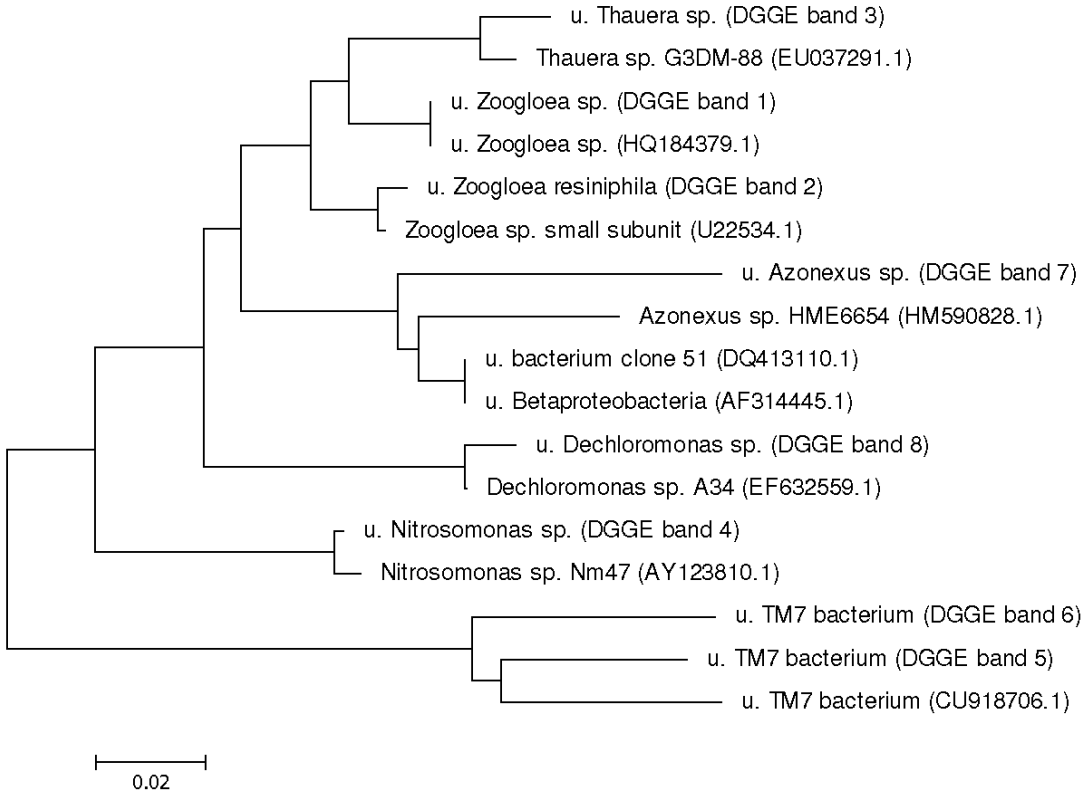


Fig. 5.8 Phylogenetic tree of the 16S rRNA gene sequences from DGGE band fragments that became intensified in the sample of R1 (ERY-H_2O), and R2 (ERY) relative to R3 (control) were constructed with the neighbor-jointing method ordination of p-distance by software MEGA 4. GeneBank accession numbers of sequences are given in *parenthesis.* And genetic similarity is above 95% between the gene detected in this study and each corresponding sequence, except that uncultured *Azonexus* detected in DGGE band 7 is 88% similar to its corresponding sequences

5.5 Discussion

Investigations on the influence of sub-inhibitory concentrations of antibiotics and their derivatives on the microbial communities of WWTPs are important due to the intimate contact of wastewater and sewage sludge with people via wastewater reclamation processes and fertilizer application, respectively (Ding and He, 2010; Le-Minh et al., 2010). On the other hand, the investigations are increasingly difficult since the influence is less pronounced due to the low concentrations of these compounds in WWTPs. In order to make influence of ERY (100 µg/L) and ERY-

H_2O (50 µg/L) detectable in SBRs, this study attained a good baseline from negative control SBR that was antibiotics-uncontaminated, explored SBRs that excluded external input of antibiotic resistant bacteria and resistance genes in case of masking the effects of antibiotics themselves, and treated microbial consortia of SBRs with ERY or ERY-H_2O for long time (over one year). Moreover, various uncultured-methods, especially high-density microarray (PhyloChip), were used in this study to give a comprehensive picture of the microbial communities. Compared to culture-based methods, the microarray technique has often been proved to be potent to profile microbial diversities in higher richness (DeSantis et al., 2007; DeSantis et al., 2005; Hazen et al., 2010), and we found this was also true in this study.

As expected, all these efforts worked together to first time display the pronounced influence of low concentrations of ERY and ERY-H_2O on the microbial communities in SBRs. Both ERY and ERY-H_2O at low concentrations (µg/L level) were found to lead to the microbial community shift as revealed by the NMDS result that the microbial communities formed two distinct clusters responding to the presence of ERY and ERY-H_2O (Fig.5.1). And the PhyloChip analysis showed that the two microbial communities shifted at a similar trend that taxonomic richness was lower and abundance was decreased in the similar inhibitory spectrum of Gram-negative Proteobacteria and Gram-positive Actinobacteria, whereas ERY-H_2O posed less considerably inhibitory effects on microbes than ERY did (Fig. 5.2–5.6 and table 5.1–5.4). The findings recall the concern on the unfavorable influence of antibiotic derivatives in the environment, although the derivatives were usually reported to possess negligible antibacterial activity due to the destruction or modification of antibiotic functional groups (Schlunzen et al., 2001; Wright, 2005). The similarly but less significantly inhibitory effects of antibiotic derivatives compared to their parent

antibiotics may act as vaccines for bacteria to build resistance more easily to the parent drugs, which may be more harmful to the human health than antibiotics themselves present in the environment. This has been confirmed in the previous studies that ERY-H_2O is able to induce microbial resistance to ERY (Fan et al., 2009; Majer, 1981). All these findings highlight that antibiotic derivatives (e.g., ERY-H_2O) may have equally vital inhibitory effects on natural microorganisms as their original antibiotics. Further study is needed to focus on both antibiotics and their derivatives in the environment.

Although inhibited microbes were discovered in the microbial communities exposed to external pressure (ERY and ERY-H_2O), there is the lack of information on the bacteria selected by ERY and ERY-H_2O. Here, PhyloChip microarray that can only give microbial community information in the subfamily level may lose the information of selected bacteria in low-level taxonomic rank (e.g., genus). Therefore, DGGE that can differentiate microbes with only one base variation was used in this study to target intensified bacteria in the community, rather than to target inhibited bacteria since DGGE is weak in targeting less dominant component bacteria. The results of DGGE, as expected, provide a picture that the presence of ERY and ERY-H_2O can lead similar enriched-bacteria spectrum, with common enriched-bacteria from the phylum of TM7 candidate division and the subphylum of Betaproteobacteria within the families of Rhodocyclaceae (*Zoogloea*, *Azonexus*) and Notrosomonadaceae (*Nitrosomonas*), and with ERY-enriched *Dechloromonas* and ERY-H_2O-enriched *Thauera* that both belonged to the family of Rhodocyclaceae (Fig. 5.7 and 5.8). *Zoogloea* is vital in maintaining activated sludge floc through excretion of EPS. Bacteria in floc or biofilm can increase antibiotic resistance 10 to 1,000 folds comparing to their planktonic formation via various mechanisms, one of which is

formation of microenvironment within biofilm that is lack of nutrient and oxygen and results in higher resistant bacteria with limited growth but enhanced anaerobic elimination of toxic intermediates (Anderson and O'Toole, 2008). For example, antibiotics triggered *Pseudomonas aeruginosa* biofilm can carry out anaerobic respiration of NO_3^- and NO_2^- into N_2 to prevent accumulation of nitric oxide through genes *nar* , *nir* , *nor* , and *nos* that are regulated by quorum sensing gene *rhlR* (Hassett et al., 2002). In this study, those enriched bacteria due to presence of ERY and ERY-H_2O may process enhanced ability to remove nitrate and ammonium similarly as shown in the above study, since most members of the genera *Azoarcus*, *Thauera* and *Dechloromonas* are reported to proceed the anaerobic reduction of nitrate coupled with degradation of anthropogenic compounds, and *Nitrosomonas* are well-known ammonium oxidizer (Loy et al., 2005). Further study is needed to clarify the genetic regulation of these biofilm antibiotic resistance mechanisms.

Noteworthy, even bacteria belonging to same genus (e.g., *Zoogloea* and *Nitrosomonas*) were affected differently by spiked ERY and ERY-H_2O, either inhibition or enrichment. The contrary effects of ERY and ERY-H_2O may be offset in the higher subfamily level targeted by the PhyloChip, which may explain why the PhyloChip analysis can not discover selected bacteria. The finding also indicate that functionally redundant groups in the complex microbial communities are important to sustain system stable under the selection of antibiotics. This is consistant with the findings in the chapter 3 that nitrifying bacteria (AOMs and NOBs) in all three SBRs had the same nitrification performance (Fig. 3.2, Fig. 3.3 and Fig. 3.4) although their diversity and total intensity were lower in R1 and R2 compared to R3, because the antibiotics-selected nitrifying bacteria may have a higher specific nitrification capacity (Table 3.1 and Fig. 3.6). In full-scale WWTPs with more complex microbial

communities, the combined effects of multiple antibiotics and their derivatives are expected different from single antibiotic influence applied on simple lab-scale SBR microbes (Ding and He, 2010). Future work is needed to study the mutiplex antibiotic influence on the microbial communities of WWTPs.

5.6 Conclusions

In summary, exposure to μg/L levels of ERY and ERY-H_2O significantly shifted microbial communities at similar ways that the diversity and abundance of bacteria decreased, especially on Gram-negative α-, γ- and δ- Proteobacteria and Gram-positive Actinobacteria, but that biofilm antibiotic resistant β-proteobacteria within the families of Rhodocyclaceae and Notrosomonadaceae were selected. This study will provide important information to substantiate the influence of antibiotics and their derivatives at sub-inhibitory concentrations on the inhibition of sensitive bacteria and the proliferation of resistant bacteria.

Chapter 6

Conclusions and recommendations

6.1 Conclusions

The lack of knowledge on the roles of antibiotics and antibiotic resistance genes in the environment has hampered efforts to prevent and control the proliferation of antibiotic resistance. As such, there is a need to study antibiotic influence on WWTPs, which are the main collection pools of anthropogenic discharges of antibiotics and antibiotic resistance. To address this need, the influence of antibiotics ERY and its derivative ERY-H_2O at µg/L levels was studied on the micro-ecosystem of lab-scale SBRs, in terms of ecological function disturbance, resistance selection and phylogenetic structure alteration. The results revealed that at low concentrations, both ERY and ERY-H_2O shared similar spectrums of sensitive bacteria and resistant bacteria. The enrichment of the resistant bacteria include those are capable of esterifying ERY via esterase *ere*A gene, as well as those are capable of forming biofilm and consequently enhanced the elimination of toxic nitrogenous substances. All these may offset the inhibitory effects of ERY and ERY-H_2O on the SBR microorganisms. The followings are important conclusions attained in this study.

1. This doctoral study has demonstrated that ERY-H_2O (50 µg/L) and ERY (100 µg/L) revealed no significant impact on the carbon and nutrients (N and P) removal efficiency of SBRs in treating the synthetic wastewater.
2. However, both ERY-H_2O (50 µg/L) and ERY (100 µg/L) can cause the composition changes of the microbial communities associated with the N and P removal in the reactors, leading to the selection of the microorganisms more resistant to ERY and ERY-H_2O.
3. This study suggests that the influence of ERY and ERY-H_2O at the µg/L levels is more likely to induce a pool of ERY resistant bacteria than to inhibit the treatment of systems (e.g., WWTPs).

4. This study also demonstrated that both ERY-H_2O (50 μg/L) and ERY (100 μg/L) can encourage the development of ERY esterase gene *ere*A in the conditions of free of continuous input of resistant bacteria and genes.
5. ERY acclimated microbes can esterify ERY via *ere*A gene, whereas ERY-H_2O acclimated ones cannot do as ERY acclimated ones, which may be due to the less proliferated *ere*A gene. Esterification of ERY required the presence of exogenous carbon source (e.g., glucose) and nutrients (e.g., nitrogen, phosphorus) for assimilation, but overdosed ammonium–N (>40 mg/L) inhibited degradation of ERY.
6. *Zoogloea*, a type of biofilm-forming bacteria, became predominant in the ERY esterification consortia, suggesting that the input of ERY could induce biofilm resistance to antibiotics.
7. Low concentrations of ERY and ERY-H_2O in the environment can result in the proliferation of antibiotic resistance genes. This study also provides important information to substantiate the correlation between the proliferation of antibiotic resistance and the antibiotics at sub-inhibitory concentrations.
8. Finally, this study demonstrated that both ERY and ERY-H_2O significantly shifted the microbial communities in similar ways.
9. It was found that Gram-negative α-, γ- and δ-Proteobacteria and the Gram-positive Actinobacteria were inhibited by both ERY and ERY-H_2O, but in different extent that ERY-H_2O posed less considerably inhibitory effects on microbes than ERY did.
10. The β-proteobacteria within the families of Rhodocyclaceae (*Azoarcus*, *Thauera*, *Dechloromonas* and *zoogloea*) and Notrosomonadaceae (*Nitrosomonas*) were enriched by both ERY and ERY-H_2O. The enrichment

resulted in the increase of antibiotic resistance due to the formation of biofilm by the *zoogloea*, the enhancement of ammonium oxidization by the *Nitrosomonas*, and the improvement of nitrate reduction by the *Azoarcus*, *Dechloromonas* and *Thauera*.

11. This study offers insights on the influence of sub-inhibitory concentrations of antibiotics and their derivatives on antibiotic sensitive and resistant bacteria.

In summary, it was found that even low concentrations of antibiotics and their derivatives were able to induce resistance genes, form antibiotic resistant biofilm and select resistant bacteria.

6.2 Recommendations

In the course of studying the influence of antibiotics ERY and its derivative ERY-H_2O on the microbial communities of lab-scale SBRs, there remain many important questions that require future research.

In chapter 3, the PhyloChip results on PAOs and GAOs were insufficient to explain the slightly improved phosphorus removal in ERY-H_2O-spiked R1 (than ERY-spiked R2 and control reactor R3). This is mainly because PAOs and GAOs include largely uncertain phylotypes that are not targeted by the PhyloChip used in this study (Seviour et al., 2003). Therefore, more information is needed in the future study. The new generation PhyloChip G3 that targets more PAOs and GAOs can be used to identify the bacteria related phosphorus removal in WWTPs.

Higher concentrations of ammonium (NH_4^+–N>40 mg/L) were found to inhibit ERY biodegradation for more than 30% (chapter 4). The even higher concentrations of ammonium in pharmaceutical wastewater will definitely make ERY biodegradation more difficult to occur. The untreated ERY, in turn, will inhibit

ammonium oxidization. Awareness is needed to optimize WWTPs to cope with this problem.

The intermediates of antibiotics may induce microbial resistance to the parent drugs (Fan et al., 2009; Majer, 1981). In chapter 4, study on ERY-H_2O confirmed the above resistance development principle on resistance gene *ere*A. Future study may need to find out whether the degradation products of other antibiotics can still induce resistance to their parent drugs as ERY-H_2O does to ERY (Fan et al., 2009; Majer, 1981).

In chapter 5, ERY and ERY-H_2O at low concentrations (μg/L level) can shift microbial communities at the similar trend, and they both can induce biofilm antibiotic resistance (Fajardo and Martinez, 2008). All these findings indicate that antibiotic derivatives (e.g., ERY-H_2O) may have the equally vital effects on microorganisms as their original antibiotics. Future study may need to focus on both antibiotics and their derivatives in the environment, to clarify their roles in the development of antibiotic resistant bacteria, and to identify gene-based biofilm antibiotic resistance (e.g., genetic regulators involved in biofilm formation).

The presence of antibiotics and their derivatives in the intestine of animals and human beings may also induce the formation of biofilm in such environments as those do in WWTPs. Inside the intestinal biofilm, the accumulated toxic compound NO_2^- may combine with a second amine to form nitrosamine, which is a carcinogen and may be harmful to the animals and human beings (Huang et al., 1996). Therefore, concerns will be raised on the unfavorable influence of antibiotics applied in the disease treatment and further study should be carried out to assess the impacts of antibiotics on the treatment of cancer patients.

Noteworthy, in chapter 5, even bacteria belonging to same genus (e.g., *Zoogloea*) were affected differently by spiked ERY and ERY-H_2O, either inhibited or enriched. This indicates that functionally redundant groups that are present in the complex microbial communities are important to sustain the stability of micro-systems under the selection pressure of antibiotics. In addition, WWTPs receive multiple antibiotics and their derivatives, and their combined effects are expected to be different from single antibiotic influence applied on microbes (Ding and He, 2010). Future work should focus more on the influence of mutiple antibiotics on complex microbial communities of real WWTPs than on relative simple lab-scale SBR fed with single ERY or ERY-H_2O.

References

Abuin, S., Codony, R., Compano, R., Granados, M., Prat, M.D., 2006. Analysis of macrolide antibiotics in river water by solid-phase extraction and liquid chromatography-mass spectrometry. Journal of Chromatography A 1114(1), 73-81.

Adelowo, O.O., Fagade, O.E., Oke, A.J., 2008. Prevalence of co-resistance to disinfectants and clinically relevant antibiotics in bacterial isolates from three hospital laboratory wastewaters in Southwestern Nigeria. World Journal of Microbiology & Biotechnology 24(9), 1993-1997.

Alexy, R., Kumpel, T., Kummerer, K., 2004. Assessment of degradation of 18 antibiotics in the Closed Bottle Test. Chemosphere 57(6), 505-512.

Alexy, R., Sommer, A., Lange, F.T., Kummerer, K., 2006. Local use of antibiotics and their input and fate in a small sewage treatment plant - significance of balancing and analysis on a local scale vs. nationwide scale. Acta Hydrochimica Et Hydrobiologica 34(6), 587-592.

Amann, R.I., Ludwig, W., Schleifer, K.H., 1995. Phylogenetic identification and in-situ detection of individual microbial-cells without cultivation. Microbiological Reviews 59(1), 143-169.

Amin, M.M., Zilles, J.L., Greiner, J., Charbonneau, S., Raskin, L., Morgenroth, E., 2006. Influence of the antibiotic erythromycin on anaerobic treatment of a pharmaceutical wastewater. Environmental Science & Technology 40(12), 3971-3977.

Aminov, R.I., 2009. The role of antibiotics and antibiotic resistance in nature. Environmental Microbiology 11(12), 2970-2988.

Anderson, G.G., O'Toole, G.A., 2008. Bacterial Biofilms, pp. 85-105, Springer-Verlag Berlin, Berlin.

Andersson, D.I., 2006. The biological cost of mutational antibiotic resistance: any practical conclusions? Current Opinion in Microbiology 9(5), 461-465.

Batt, A.L., Kim, S., Aga, D.S., 2006. Enhanced biodegradation of iopromide and trimethoprim in nitrifying activated sludge. Environmental Science & Technology 40(23), 7367-7373.

Batt, A.L., Kim, S., Aga, D.S., 2007. Comparison of the occurrence of antibiotics in four full-scale wastewater treatment plants with varying designs and operations. Chemosphere 68(3), 428-435.

Beausse, J., 2004. Selected drugs in solid matrices: a review of environmental determination, occurrence and properties of principal substances. Trac-Trends in Analytical Chemistry 23(10-11), 753-761.

Bendz, D., Paxeus, N.A., Ginn, T.R., Loge, F.J., 2005. Occurrence and fate of pharmaceutically active compounds in the environment, a case study: Hoje River in Sweden. Journal of Hazardous Materials 122(3), 195-204.

Brodie, E.L., DeSantis, T.Z., Joyner, D.C., Baek, S.M., Larsen, J.T., Andersen, G.L., Hazen, T.C., Richardson, P.M., Herman, D.J., Tokunaga, T.K., Wan, J.M.M., Firestone, M.K., 2006. Application of a high-density oligonucleotide microarray approach to study bacterial population dynamics during uranium reduction and reoxidation. Applied and Environmental Microbiology 72(9), 6288-6298.

Brown, K.D., Kulis, J., Thomson, B., Chapman, T.H., Mawhinney, D.B., 2006. Occurrence of antibiotics in hospital, residential, and dairy, effluent, municipal wastewater, and the Rio Grande in New Mexico. Science of the Total Environment 366(2-3), 772-783.

Calamari, D., Zuccato, E., Castiglioni, S., Bagnati, R., Fanelli, R., 2003. Strategic survey of therapeutic drugs in the rivers Po and Lambro in northern Italy. Environmental Science & Technology 37(7), 1241-1248.

Carballa, M., Omil, F., Alder, A.C., Lema, J.M., 2006. Comparison between the conventional anaerobic digestion of sewage sludge and its combination with a chemical or thermal pre-treatment concerning the removal of pharmaceuticals and personal care products. Water Science and Technology 53(8), 109-117.

Carballa, M., Omil, F., Lema, J.M., Llompart, M., Garcia-Jares, C., Rodriguez, I., Gomez, M., Ternes, T., 2004. Behavior of pharmaceuticals, cosmetics and hormones in a sewage treatment plant. Water Research 38(12), 2918-2926.

Carballa, M., Omil, F., Lema, J.M., Llompart, M., Garcia, C., Rodriguez, I., Gomez, M., Ternes, T., 2005. Behaviour of pharmaceuticals and personal care products in a sewage treatment plant of northwest Spain. Water Science and Technology 52(8), 29-35.

Cha, J.M., Yang, S., Carlson, K.H., 2006. Trace determination of beta-lactam antibiotics in surface water and urban wastewater using liquid chromatography combined with electrospray tandem mass spectrometry. Journal of Chromatography A 1115(1-2), 46-57.

Choi, K.J., Kim, S.G., Kim, C.W., Kim, S.H., 2007. Determination of antibiotic compounds in water by on-line SPE-LC/MSD. Chemosphere 66(6), 977-984.

Christian, T., Schneider, R.J., Farber, H.A., Skutlarek, D., Meyer, M.T., Goldbach, H.E., 2003. Determination of antibiotic residues in manure, soil, and surface waters. Acta Hydrochimica Et Hydrobiologica 31(1), 36-44.

Clara, M., Strenn, B., Gans, O., Martinez, E., Kreuzinger, N., Kroiss, H., 2005. Removal of selected pharmaceuticals, fragrances and endocrine disrupting compounds in a membrane bioreactor and conventional wastewater treatment plants. Water Research 39(19), 4797-4807.

Costanzo, S.D., Murby, J., Bates, J., 2005. Ecosystem response to antibiotics entering the aquatic environment. Marine Pollution Bulletin 51(1-4), 218-223.

Daughton, C.G., 2004. Non-regulated water contaminants: emerging research. Environmental Impact Assessment Review 24(7-8), 711-732.

Daughton, C.G., Ternes, T.A., 1999. Pharmaceuticals and personal care products in the environment: Agents of subtle change? Environmental Health Perspectives 107, 907-938.

Davies, J., Davies, D., 2010. Origins and Evolution of Antibiotic Resistance. Microbiology and Molecular Biology Reviews 74(3), 417-+.

Davies, J., Spiegelman, G.B., Yim, G., 2006. The world of subinhibitory antibiotic concentrations. Current Opinion in Microbiology 9(5), 445-453.

DeSantis, T.Z., Brodie, E.L., Moberg, J.P., Zubieta, I.X., Piceno, Y.M., Andersen, G.L., 2007. High-density universal 16S rRNA microarray analysis reveals broader diversity than typical clone library when sampling the environment. Microbial Ecology 53(3), 371-383.

DeSantis, T.Z., Dubosarskiy, I., Murray, S.R., Andersen, G.L., 2003. Comprehensive aligned sequence construction for automated design of effective probes (CASCADE-P) using 16S rDNA. Bioinformatics 19(12), 1461-1468.

DeSantis, T.Z., Stone, C.E., Murray, S.R., Moberg, J.P., Andersen, G.L., 2005. Rapid quantification and taxonomic classification of environmental DNA from both prokaryotic and eukaryotic origins using a microarray. FEMS Microbiology Letters 245(2), 271-278.

Deshpande, A.D., Baheti, K.G., Chatterjee, N.R., 2004. Degradation of beta-lactam antibiotics. Current Science 87(12), 1684-1695.

Diaz-Cruz, M.S., de Alda, M.J.L., Barcelo, D., 2003. Environmental behavior and analysis of veterinary and human drugs in soils, sediments and sludge. Trac-Trends in Analytical Chemistry 22(6), 340-351.

Ding, C., He, J.Z., 2010. Effect of antibiotics in the environment on microbial populations. Applied Microbiology and Biotechnology 87(3), 925-941.

Dubois, M., Fluchard, D., Sior, E., Delahaut, P., 2001. Identification and quantification of five macrolide antibiotics in several tissues, eggs and milk by liquid chromatography-electrospray tandem mass spectrometry. Journal of Chromatography B 753(2), 189-202.

Enne, V.I., Bennett, P.M., Livermore, D.M., Hall, L.M.C., 2004. Enhancement of host fitness by the sul2-coding plasmid p9123 in the absence of selective pressure. Journal of Antimicrobial Chemotherapy 53(6), 958-963.

Enne, V.I., Delsol, A.A., Davis, G.R., Hayward, S.L., Roe, J.M., Bennett, P.M., 2005. Assessment of the fitness impacts on Escherichia coli of acquisition of

antibiotic resistance genes encoded by different types of genetic element. Journal of Antimicrobial Chemotherapy 56(3), 544-551.

Fajardo, A., Martinez, J.L., 2008. Antibiotics as signals that trigger specific bacterial responses. Current Opinion in Microbiology 11(2), 161-167.

Fan, C., He, J., 2011. Proliferation of antibiotic resistance genes in microbial consortia of sequencing batch reactors (SBRs) upon exposure to trace erythromycin or erythromycin-H_2O. Water Research 45(10), 3098-3106.

Fan, C.A., Lee, P.K.H., Ng, W.J., Alvarez-Cohen, L., Brodie, E.L., Andersen, G.L., He, J.Z., 2009. Influence of trace erythromycin and erythromycin-H_2O on carbon and nutrients removal and on resistance selection in sequencing batch reactors (SBRs). Applied Microbiology and Biotechnology 85(1), 185-195.

Ferrer, I., Thurman, E.M., 2003. Liquid chromatography/time-of-flight/mass spectrometry (LC/TOF/MS) for the analysis of emerging contaminants. Trac-Trends in Analytical Chemistry 22(10), 750-756.

Flanagan, J.L., Brodie, E.L., Weng, L., Lynch, S.V., Garcia, O., Brown, R., Hugenholtz, P., DeSantis, T.Z., Andersen, G.L., Wiener-Kronish, J.P., Bristow, J., 2007. Loss of bacterial diversity during antibiotic treatment of intubated patients colonized with Pseudomonas aeruginosa. Journal of Clinical Microbiology 45(6), 1954-1962.

Focazio, M.J., Kolpin, D.W., Barnes, K.K., Furlong, E.T., Meyer, M.T., Zaugg, S.D., Barber, L.B., Thurman, M.E., 2008. A national reconnaissance for pharmaceuticals and other organic wastewater contaminants in the United States - II) Untreated drinking water sources. Science of the Total Environment 402(2-3), 201-216.

Forth, W., Henschler, D., Rummel, W., Starke, K., 1992. Allgemeine und spezielle Pharmakologie und Toxikologie, BIWissenschaftsverlag, Mannheim, Leipzig, Wien, Zürich.

Fountoulakis, M., Drillia, P., Stamatelatou, K., Lyberatos, G., 2004. Tonic effect of pharmaceuticals on methanogienesis. Water Science and Technology 50(5), 335-340.

Fountoulakis, M.S., Stamatelatou, K., Lyberatos, G., 2008. The effect of pharmaceuticals on the kinetics of methanogenesis and acetogenesis. Bioresource Technology 99(15), 7083-7090.

Gartiser, S., Urich, E., Alexy, R., Kummerer, K., 2007a. Anaerobic inhibition and biodegradation of antibiotics in ISO test schemes. Chemosphere 66(10), 1839-1848.

Gartiser, S., Urich, E., Alexy, R., Kummerer, K., 2007b. Ultimate biodegradation and elimination of antibiotics in inherent tests. Chemosphere 67(3), 604-613.

Giger, W., Alder, A.C., Golet, E.M., Kohler, H.P.E., McArdell, C.S., Molnar, E., Siegrist, H., Suter, M.J.F., 2003. Occurrence and fate of antibiotics as trace

contaminants in wastewaters, sewage sludges, and surface waters. Chimia 57(9), 485-491.

Gobel, A., McArdell, C.S., Joss, A., Siegrist, H., Giger, W., 2007. Fate of sulfonamides, macrolides, and trimethoprim in different wastewater treatment technologies. Science of the Total Environment 372(2-3), 361-371.

Gobel, A., Thomsen, A., McArdell, C.S., Joss, A., Giger, W., 2005. Occurrence and sorption behavior of sulfonamides, macrolides, and trimethoprim in activated sludge treatment. Environmental Science & Technology 39(11), 3981-3989.

Golet, E.M., Xifra, I., Siegrist, H., Alder, A.C., Giger, W., 2003. Environmental exposure assessment of fluoroquinolone antibacterial agents from sewage to soil. Environmental Science & Technology 37(15), 3243-3249.

Gomez, J., Mendez, R., Lema, J.M., 1996. The effect of antibiotics on nitrification processes - Batch assays. Applied Biochemistry and Biotechnology 57-8, 869-876.

Gros, M., Petrovic, M., Barcelo, D., 2006. Development of a multi-residue analytical methodology based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) for screening and trace level determination of pharmaceuticals in surface and wastewaters. Talanta 70(4), 678-690.

Gros, M., Petrovic, M., Barcelo, D., 2007. Wastewater treatment plants as a pathway for aquatic contamination by pharmaceuticals in the Ebro river basin (Northeast Spain). Environmental Toxicology and Chemistry 26(8), 1553-1562.

Gulkowska, A., Leung, H.W., So, M.K., Taniyasu, S., Yamashita, N., Yeunq, L.W.Y., Richardson, B.J., Lei, A.P., Giesy, J.P., Lam, P.K.S., 2008. Removal of antibiotics from wastewater by sewage treatment facilities in Hong Kong and Shenzhen, China. Water Research 42(1-2), 395-403.

Halling-Sorensen, B., 2001. Inhibition of aerobic growth and nitrification of bacteria in sewage sludge by antibacterial agents. Archives of Environmental Contamination and Toxicology 40(4), 451-460.

Hancock, R.E.W., 2005. Mechanisms of action of newer antibiotics for Gram-positive pathogens. Lancet Infectious Diseases 5(4), 209-218.

Harrison, J.J., Ceri, H., Roper, N.J., Badry, E.A., Sproule, K.M., Turner, R.J., 2005. Persister cells mediate tolerance to metal oxyanions in Escherichia coli. Microbiology-Sgm 151, 3181-3195.

Hassett, D.J., Cuppoletti, J., Trapnell, B., Lymar, S.V., Rowe, J.J., Yoon, S.S., Hilliard, G.M., Parvatiyar, K., Kamani, M.C., Wozniak, D.J., Hwang, S.H., McDermott, T.R., Ochsner, U.A., 2002. Anaerobic metabolism and quorum sensing by Pseudomonas aeruginosa biofilms in chronically infected cystic fibrosis airways: rethinking antibiotic treatment strategies and drug targets. Advanced Drug Delivery Reviews 54(11), 1425-1443.

Hazen, T.C., Dubinsky, E.A., DeSantis, T.Z., Andersen, G.L., Piceno, Y.M., Singh, N., Jansson, J.K., Probst, A., Borglin, S.E., Fortney, J.L., Stringfellow, W.T., Bill, M., Conrad, M.E., Tom, L.M., Chavarria, K.L., Alusi, T.R., Lamendella, R., Joyner, D.C., Spier, C., Baelum, J., Auer, M., Zemla, M.L., Chakraborty, R., Sonnenthal, E.L., D'Haeseleer, P., Holman, H.Y.N., Osman, S., Lu, Z.M., Van Nostrand, J.D., Deng, Y., Zhou, J.Z., Mason, O.U., 2010. Deep-Sea Oil Plume Enriches Indigenous Oil-Degrading Bacteria. Science 330(6001), 204-208.

He, J.Z., Ritalahti, K.M., Yang, K.L., Koenigsberg, S.S., Loffler, F.E., 2003. Detoxification of vinyl chloride to ethene coupled to growth of an anaerobic bacterium. Nature 424(6944), 62-65.

Hirsch, R., Ternes, T., Haberer, K., Kratz, K.L., 1999. Occurrence of antibiotics in the aquatic environment. Science of the Total Environment 225(1-2), 109-118.

Huang, Y.G., Ji, J.D., Hou, Q.N., 1996. A study on carcinogenesis of endogenous nitrite and nitrosamine, and prevention of cancer. Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis 358(1), 7-14.

Ingerslev, F., Halling-Sorensen, B., 2000. Biodegradability properties of sulfonamides in activated sludge. Environmental Toxicology and Chemistry 19(10), 2467-2473.

Jones, O.A.H., Voulvoulis, N., Lester, J.N., 2007. The occurrence and removal of selected pharmaceutical compounds in a sewage treatment works utilising activated sludge treatment. Environmental Pollution 145(3), 738-744.

Karthikeyan, K.G., Meyer, M.T., 2006. Occurrence of antibiotics in wastewater treatment facilities in Wisconsin, USA. Science of the Total Environment 361(1-3), 196-207.

Kim, S., Eichhorn, P., Jensen, J.N., Weber, A.S., Aga, D.S., 2005. Removal of antibiotics in wastewater: Effect of hydraulic and solid retention times on the fate of tetracycline in the activated sludge process. Environmental Science & Technology 39(15), 5816-5823.

Kim, S., Jensen, J.N., Aga, D.S., Weber, A.S., 2007. Tetracycline as a selector for resistant bacteria in activated sludge. Chemosphere 66(9), 1643-1651.

Kim, Y.H., Heinze, T.M., Beger, R., Pothuluri, J.V., Cerniglia, C.E., 2004. A kinetic study on the degradation of erythromycin A in aqueous solution. International Journal of Pharmaceutics 271(1-2), 63-76.

Klaver, A.L., Matthews, R.A., 1994. Effects of oxytetracycline on nitrification in a model aquatic system. Aquaculture 123(3-4), 237-247.

Koch, G., Kuhni, M., Siegrist, H., 2001. Calibration and validation of an ASM3-based steady-state model for activated sludge systems - Part I: prediction of nitrogen removal and sludge production. Water Research 35(9), 2235-2245.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999-2000: A national reconnaissance. Environmental Science & Technology 36(6), 1202-1211.

Kummerer, K., 2009a. Antibiotics in the aquatic environment - A review - Part I. Chemosphere 75(4), 417-434.

Kummerer, K., 2009b. Antibiotics in the aquatic environment - A review - Part II. Chemosphere 75(4), 435-441.

Lane, D.J., Pace, B., Olsen, G.J., Stahl, D.A., Sogin, M.L., Pace, N.R., 1985. Rapid-determination of 16S ribosomal-RNA sequences for phylogenetic analyses. Proceedings of the National Academy of Sciences of the United States of America 82(20), 6955-6959.

Le-Minh, N., Khan, S.J., Drewes, J.E., Stuetz, R.M., 2010. Fate of antibiotics during municipal water recycling treatment processes. Water Research 44(15), 4295-4323.

Lenski, R.E., 1997. Antibiotic Resistance: Origins, Evolution, Selection and Spread. Chadwick, D.J.,Goode, J. (eds), pp. 131-140, John Wiley & Sons Ltd, Chichester.

Li, X.Y., Yang, S.F., 2007. Influence of loosely bound extracellular polymeric substances (EPS) on the flocculation, sedimentation and dewaterability of activated sludge. Water Research 41(5), 1022-1030.

Lin, A.Y.C., Tsai, Y.T., 2009. Occurrence of pharmaceuticals in Taiwan's surface waters: Impact of waste streams from hospitals and pharmaceutical production facilities. Science of the Total Environment 407(12), 3793-3802.

Lin, A.Y.C., Yu, T.H., Lateef, S.K., 2009. Removal of pharmaceuticals in secondary wastewater treatment processes in Taiwan. Journal of Hazardous Materials 167(1-3), 1163-1169.

Linares, J.F., Gustafsson, I., Baquero, F., Martinez, J.L., 2006. Antibiotics as intermicrobial signaling agents instead of weapons. Proceedings of the National Academy of Sciences of the United States of America 103(51), 19484-19489.

Lindberg, R.H., Wennberg, P., Johansson, M.I., Tysklind, M., Andersson, B.A.V., 2005. Screening of human antibiotic substances and determination of weekly mass flows in five sewage treatment plants in Sweden. Environmental Science & Technology 39(10), 3421-3429.

Lipsitch, M., Samore, M.H., 2002. Antimicrobial use and antimicrobial resistance: A population perspective. Emerging Infectious Diseases 8(4), 347-354.

Loy, A., Schulz, C., Lucker, S., Schopfer-Wendels, A., Stoecker, K., Baranyi, C., Lehner, A., Wagner, M., 2005. 16S rRNA gene-based oligonucleotide microarray for environmental monitoring of the betaproteobacterial order

"Rhodocyclales". Applied and Environmental Microbiology 71(3), 1373-1386.

Luo, N.D., Pereira, S., Sahin, O., Lin, J., Huang, S.X., Michel, L., Zhang, Q.J., 2005. Enhanced in vivo fitness of fluoroquinolone-resistant Campylobacter jejuni in the absence of antibiotic selection pressure. Proceedings of the National Academy of Sciences of the United States of America 102(3), 541-546.

Majer, J., 1981. In vitro introduction of resistance to erythromycin by its metabolite. Antimicrobial Agents and Chemotherapy 19(4), 628-633.

Martinez, J.L., 2008. Antibiotics and antibiotic resistance genes in natural environments. Science 321(5887), 365-367.

McArdell, C.S., Molnar, E., Suter, M.J.F., Giger, W., 2003. Occurrence and fate of macrolide antibiotics in wastewater treatment plants and in the Glatt Valley Watershed, Switzerland. Environmental Science & Technology 37(24), 5479-5486.

Miao, X.S., Bishay, F., Chen, M., Metcalfe, C.D., 2004. Occurrence of antimicrobials in the final effluents of wastewater treatment plants in Canada. Environmental Science & Technology 38(13), 3533-3541.

Morar, M., Wright, G.D., 2010. Annual Review of Genetics, Vol 44, pp. 25-51.

Murray, B.E., 1997. Advances in Internal Medicine, Vol 42, pp. 339-367, Mosby-Year Book Inc, St Louis.

Muyzer, G., Dewaal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial-populations by denaturing gradient gel-electrophoresis analysis of polymerase chain reaction-amplified genes-coding for 16S ribosomal-RNA. Applied and Environmental Microbiology 59(3), 695-700.

Nagulapally, S.R., Ahmad, A., Henry, A., Marchin, G.L., Zurek, L., Bhandari, A., 2009. Occurrence of Ciprofloxacin-, Trimethoprim-Sulfamethoxazole-, and Vancomycin-Resistant Bacteria in a Municipal Wastewater Treatment Plant. Water Environment Research 81(1), 82-90.

Nimenya, H., Delaunois, A., La Duong, D., Bloden, S., Defour, J., Nicks, B., Ansay, M., 1999. Short-term toxicity of various pharmacological agents on the in vitro nitrification process in a simple closed aquatic system. ATLA Alternatives to Laboratory Animals 27(1), 121-135.

Ohlsen, K., Ternes, T., Werner, G., Wallner, U., Loffler, D., Ziebuhr, W., Witte, W., Hacker, J., 2003. Impact of antibiotics on conjugational resistance gene transfer in Staphylococcus aureus in sewage. Environmental Microbiology 5(8), 711-716.

Ohlsen, K., Ziebuhr, W., Koller, K.P., Hell, W., Wichelhaus, T.A., Hacker, J., 1998. Effects of subinhibitory concentrations of antibiotics on alpha-toxin (hla) gene expression of methicillin-sensitive and methichillin-resistant

Staphylococcus aureus isolates. Antimicrobial Agents and Chemotherapy 42(11), 2817-2823.

Oleinick, N.L., 1975. Mechanism of action of antimicrobial and antitumor agents. Corcoran, J.W.,Hahn, F.E. (eds), Springer-Verlag, Berlin.

Pechere, J.C., 2001. Macrolide resistance mechanisms in Gram-positive cocci. International Journal of Antimicrobial Agents 18, S25-S28.

Peng, X.Z., Wang, Z.D., Kuang, W.X., Tan, J.H., Li, K., 2006. A preliminary study on the occurrence and behavior of sulfonamides, ofloxacin and chloramphenicol antimicrobials in wastewaters of two sewage treatment plants in Guangzhou, China. Science of the Total Environment 371(1-3), 314-322.

Perez, S., Eichhorn, P., Aga, D.S., 2005. Evaluating the biodegradability of sulfamethazine, sulfamethoxazole, sulfathiazole, and trimethoprim at different stages of sewage treatment. Environmental Toxicology and Chemistry 24(6), 1361-1367.

Pothuluri, J.V., Nawaz, M., Cerniglia, C.E., 1998. Bioremediation: Principles and Practice. Sikdar, S.K.,Irvine, R.L. (eds), pp. 221-248, Technomic Publishing Co., Lancaster, Basel.

Prosser, J.I., 1989. Autotrophic nitrification in bacteria. Advances in Microbial Physiology 30, 125-181.

Rachid, S., Ohlsen, K., Witte, W., Hacker, J., Ziebuhr, W., 2000. Effect of subinhibitory antibiotic concentrations on polysaccharide intercellular adhesin expression in biofilm-forming Staphylococcus epidermidis. Antimicrobial Agents and Chemotherapy 44(12), 3357-3363.

Reysenbach, A.L., Wickham, G.S., Pace, N.R., 1994. Phylogenetic analysis of the hyperthermophilic pink filament community in Octopus Spring, Yellowstone-National-Park. Applied and Environmental Microbiology 60(6), 2113-2119.

Richardson, B.J., Larn, P.K.S., Martin, M., 2005. Emerging chemicals of concern: Pharmaceuticals and personal care products (PPCPs) in Asia, with particular reference to Southern China. Marine Pollution Bulletin 50(9), 913-920.

Roberts, P.H., Thomas, K.V., 2006. The occurrence of selected pharmaceuticals in wastewater effluent and surface waters of the lower Tyne catchment. Science of the Total Environment 356(1-3), 143-153.

Rosal, R., Rodriguez, A., Perdigon-Melon, J.A., Petre, A., Garcia-Calvo, E., Gomez, M.J., Aguera, A., Fernandez-Alba, A.R., 2010. Occurrence of emerging pollutants in urban wastewater and their removal through biological treatment followed by ozonation. Water Research 44(2), 578-588.

Schlunzen, F., Zarivach, R., Harms, J., Bashan, A., Tocilj, A., Albrecht, R., Yonath, A., Franceschi, F., 2001. Structural basis for the interaction of antibiotics

with the peptidyl transferase centre in eubacteria. Nature 413(6858), 814-821.

Seviour, R.J., Mino, T., Onuki, M., 2003. The microbiology of biological phosphorus removal in activated sludge systems. FEMS Microbiology Reviews 27(1), 99-127.

Sutcliffe, J., Grebe, T., TaitKamradt, A., Wondrack, L., 1996. Detection of erythromycin-resistant determinants by PCR. Antimicrobial Agents and Chemotherapy 40(11), 2562-2566.

Szczepanowski, R., Linke, B., Krahn, I., Gartemann, K.H., Gutzkow, T., Eichler, W., Puhler, A., Schluter, A., 2009. Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. Microbiology-Sgm 155, 2306-2319.

Tauxe-Wuersch, A., De Alencastro, L.F., Grandjean, D., Tarradellas, J., 2005. Occurrence of several acidic drugs in sewage treatment plants in Switzerland and risk assessment. Water Research 39(9), 1761-1772.

Tomlinso.Tg, Boon, A.G., Trotman, C.N.A., 1966. Inhibition of nitrification in activated sludge process of sewage disposal. Journal of Applied Bacteriology 29(2), 266-&.

Verdier, L., Gharbi-Benarous, J., Bertho, G., Mauvais, P., Girault, J.P., 2001. A novel mechanism of antibiotic resistance: study of the complex state of peptides with bacterial *Staphylococcus aureus* ribosomes. Comptes Rendus De L Academie Des Sciences Serie Ii Fascicule C-Chimie 4(10), 745-750.

Vieno, N., Tuhkanen, T., Kronberg, L., 2007. Elimination of pharmaceuticals in sewage treatment plants in Finland. Water Research 41(5), 1001-1012.

Walsh, C., 2000. Molecular mechanisms that confer antibacterial drug resistance. Nature 406(6797), 775-781.

Watkinson, A.J., Murby, E.J., Costanzo, S.D., 2007. Removal of antibiotics in conventional and advanced wastewater treatment: Implications for environmental discharge and wastewater recycling. Water Research 41(18), 4164-4176.

Watson, S.W., Bock, E., Harms, H., Koops, H.-P., Hooper, A.B. (eds) 1989. Nitrifying bacteria, Williams and Wilkins, Baltimore, MD.

Wondrack, L., Massa, M., Yang, B.V., Sutcliffe, J., 1996. Clinical strain of Staphylococcus aureus inactivates and causes efflux of macrolides. Antimicrobial Agents and Chemotherapy 40(4), 992-998.

Wright, G.D., 2005. Bacterial resistance to antibiotics: Enzymatic degradation and modification. Advanced Drug Delivery Reviews 57(10), 1451-1470.

Wright, G.D., 2010. The antibiotic resistome. Expert Opinion on Drug Discovery 5(8), 779-788.

Xiao, Y., Chang, H., Jia, A., Hu, J.Y., 2008. Trace analysis of quinolone and fluoroquinolone antibiotics from wastewaters by liquid chromatography-electrospray tandem mass spectrometry. Journal of Chromatography A 1214(1-2), 100-108.

Xu, W.H., Zhang, G., Li, X.D., Zou, S.C., Li, P., Hu, Z.H., Li, J., 2007a. Occurrence and elimination of antibiotics at four sewage treatment plants in the Pearl River Delta (PRD), South China. Water Research 41(19), 4526-4534.

Xu, W.H., Zhang, G., Zou, S.C., Li, X.D., Liu, Y.C., 2007b. Determination of selected antibiotics in the Victoria Harbour and the Pearl River, South China using high-performance liquid chromatography-electrospray ionization tandem mass spectrometry. Environmental Pollution 145(3), 672-679.

Yang, Q., Peng, Y.Z., Liu, X.H., Zeng, W., Mino, T., Satoh, H., 2007. Nitrogen removal via nitrite from municipal wastewater at low temperatures using real-time control to optimize nitrifying communities. Environmental Science & Technology 41(23), 8159-8164.

Yang, S.W., Cha, J.M., Carlson, K., 2005. Simultaneous extraction and analysis of 11 tetracycline and sulfonamide antibiotics in influent and effluent domestic wastewater by solid-phase extraction and liquid chromatography-electro spray ionization tandem mass spectrometry. Journal of Chromatography A 1097(1-2), 40-53.

Yang, S.W., Cha, J.M., Carlson, K., 2006. Trace analysis and occurrence of anhydroerythromycin and tylosin in influent and effluent wastewater by liquid chromatography combined with electrospray tandem mass spectrometry. Analytical and Bioanalytical Chemistry 385(3), 623-636.

Yang, Y., Wang, P., Shi, S., Liu, Y., 2009. Microwave enhanced Fenton-like process for the treatment of high concentration pharmaceutical wastewater. Journal of Hazardous Materials 168(1), 238-245.

Yasojima, M., Nakada, N., Komori, K., Suzuki, Y., Tanaka, H., 2006. Occurrence of levofloxacin, clarithromycin and azithromycin in wastewater treatment plant in Japan. Water Science and Technology 53(11), 227-233.

Yim, G., Wang, H.H.M., Davies, J., 2006. The truth about antibiotics. International Journal of Medical Microbiology 296(2-3), 163-170.

Zhang, X.X., Zhang, T., Fang, H., 2009. Antibiotic resistance genes in water environment. Applied Microbiology and Biotechnology 82(3), 397-414.

Zhou, J.Z., Davey, M.E., Figueras, J.B., Rivkina, E., Gilichinsky, D., Tiedje, J.M., 1997. Phylogenetic diversity of a bacterial community determined from Siberian tundra soil DNA. Microbiology-Uk 143, 3913-3919.

Zhou, J.Z., Fries, M.R., Cheesanford, J.C., Tiedje, J.M., 1995. Phylogenetic analysis of a new group of denitrifiers capable of anaerobic growth on toluene and description of *Azoarcus- tolulyticus* sp-nov. International Journal of Systematic Bacteriology 45(3), 500-506.

Zorita, S., Martensson, L., Mathiasson, L., 2009. Occurrence and removal of pharmaceuticals in a municipal sewage treatment system in the south of Sweden. Science of the Total Environment 407(8), 2760-2770.