NANYANG TECHNOLOGICAL UNIVERSITY

REMOVAL OF ANTIBIOTICS FROM WASTEWATER BY ADSORPTION AND BIODEGRADATION

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2011

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A thesis submitted to the Nanyang Technological University

in fulfilment of the requirement for the degree of
Doctor of Philosophy

2011

ABSTRACT

Antibiotics may enter the water cycle from a variety of sources, including discharges from hospitals, domestic sewage and manufacturing processes, etc.. The occurrence of these chemicals in the aquatic environment has been reported all over the world. Recently, the adverse effects of antibiotics in water on the ecosystem and on the human health have been recognized. This means that wastewater treatment plants should take special actions to treat the antibiotic-bearing wastewater. However, research focused on the removal of antibiotics is still limited. Among existing wastewater treatment technologies, adsorption and biodegradation are both extensively used for handling a wide variety of wastewaters. Therefore, this study aimed to develop a novel combined adsorption-biodegradation process for efficiently treating antibiotic-bearing wastewater, i.e. the biofilm-covered granular activated carbon (BGAC) system.

In the first phase of study, adsorption of three typical β-lactam antibiotics, namely, penicillin G (PCG), ampicillin (AMP) and cephalosporin C (CPC) by granular activated carbon (GAC) was investigated. Characterization of the antibiotic-loaded GAC showed that PCG, AMP and CPC in solution would be first adsorbed in the microspores of GAC, and were further bound to the GAC functional groups. Results showed that Langmuir isotherm can provide the satisfactory prediction of the equilibrium data, and the maximum adsorption capacity (q_m) at 25°C was found to be 427.3, 164.2 and 33.67 mg g^{-1} for PCG, AMP and CPC, respectively. These suggest that GAC is an effective adsorbent for antibiotics. In addition, the thermodynamic analysis revealed that the adsorption of these antibiotics by GAC would be of chemisorption.

For almost all the adsorption study, empirical first- and second-order rate equations are commonly used without understanding their theoretical origins. In such a situation, a new transformed Langmuir kinetics was for the first time developed in this study, by which it was further shown that first- and second-order rate equations for adsorption would be special cases of Langmuir kinetics under certain conditions. A principle for simplification of Langmuir kinetics to first- or second-order rate equation was thus established. The proposed theory for adsorption kinetics was verified with the data of antibiotics adsorption by GAC as well as the literature data.

To exploit a high-efficiency process for treating antibiotic-bearing wastewater, a continuous airlift bioreactor with GAC as support carrier was successfully developed. It was clearly shown that the biofilm-covered GAC, namely BGAC, had the strong capability to remove AMP present in the influent. For example, the AMP concentration was not detectable in the reactor effluent during the 60-day of the continuous operation. The results indicated that AMP at the concentration studied would not affect the biofilm development, morphology and activity, while it may alter the chemical composition of biofilms. The removal mechanisms of AMP by BGAC were also examined. It was found that biosorption of AMP by the biofilms was negligible, while adsorption of AMP by GAC and biodegradation by the biofilms on GAC would be mainly responsible for the observed AMP removal. However, it was demonstrated that the AMP biodegradation by the biofilms became stronger and stronger along with the biofilm development. As such, significant bio-regeneration of BGAC was achieved in the continuous airlift bioreactor.

The advanced molecular biology techniques were employed to further examine the microbial community of the biofilms developed on GAC for AMP removal. Denaturing gradient gel electrophoresis (DGGE) analysis showed that AMP had the negative effect on the biofilm community diversity, but exerted a positive pressure on the community stability. Bacterial species were identified through 16S rRNA gene sequencing for the biofilms developed with/without AMP, and the suspended activated sludge supplemented with AMP. *Acidovorax sp.*, a typical AMP resistant bacterium, was found to be the dominant genus with an abundance of about 35% in the biofilms developed with 8 mg L^{-1} AMP. A direct correlation between the AMP biodegradation capability and the amount of AMP-degrading bacteria in the biofilms was subsequently established. In conclusion, this study clearly showed that combined adsorption and biodegradation would be a promising technology for high-efficiency treatment of the antibiotic-bearing wastewater.

ACKNOWLEDGEMENTS

First of all, I would like to express my sincere gratitude to my supervisor, Associate Professor Liu Yu for his supervision, patience and encouragement. His steady enthusiasms towards academics and strong professional knowledge have inspired me a great deal during my PhD study. This dissertation could not be completed smoothly without his prudent guidance and invaluable advice.

I want to express my special thanks to Dr. Xu Hailou, Singapore Polytechnic, for his technical assistance in identification of microbial species. My great thanks are given to all friendly staff in the Environment Laboratory, for their kind help and professional assistance in laboratory work.

My tremendous appreciation goes to School of Civil and Environmental Engineering, Nanyang Technological University, for their scholarship, facilities and heartfelt assistance.

I am indebted to my home university, Xiamen University, for its invaluable support for my PhD study in Singapore.

Finally, I wish to dedicate this piece of work to my family and all friends for their full support during my study at NTU. It is their love that has given me courage, motivation and perseverance all throughout my pursuit in research and in life.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ACN	Acetonitrile
AMP	Ampicillin
AOP	Advanced oxidation processes
BET	Brunauer-Emmett-Teller
BGAC	Biofilm-covered granular activated carbon
CBT	Closed bottle test
CFU	Colony forming unit
CLSM	Confocal laser scanning microscope
COD	Chemical oxygen demand
CPC	Cephalosporin C
CTC	Chlortetracyclin
DAPI	4'6-diamidino-2-phenylindole
DGGE	Denaturing gradient gel electrophoresis
DTGS	Deuterated triglycine sulfate
ED	Electrodialysis
EPS	Extracellular polymeric substances
FTIR	Fourier transform infrared
GAC	Granular activated carbon
HGT	Horizontal gene transfer
HPLC	High-performance liquid chromatography

HRT	Hydraulic retention time
LB	Luria-Bertani
LC-MS-MS	Liquid chromatograph-mass spectrometer-mass spectrometer
MBR	Membrane bioreactor reactor
MET	Metronidazole
MF	Microfiltration
MIC	Minimum inhibitory concentration
NF	Nanofiltration
NMDS	Non-metric multidimensional scaling
OLA	Olaquindox
OTC	Oxytetracycline
OTU	operational taxonomic units
PAC	Powdered activated carbon
PCG	Penicillin G
PCR	Polymerase chain reaction
PLFA	Phospholipid-derived fatty acids
PN	Extracellular proteins
PS	Extracellular polysaccharides
RO	Reverse osmosis
SBR	Sequencing batch reactor
SDZ	Sulfadiazine
SEM	Scanning electron microscopy

SOUR	Specific oxygen uptake rate
SPE	Solid phase extraction
TC	Tetracycline
TOC	Total organic carbon
TYL	Tylosin
UF	Ultrafiltration
UPGMA	Unweighted pair-group method using arithmetical averages
WWTP	Wastewater treatment plant

PUBLICATIONS

I: Journal Papers

1. Shen, L., Xu, H.L. and Liu, Y. (2011) Microbial characterization of the biofilms developed for treating ampicillin-bearing wastewater. Journal of Environmental Science and Health-Part A 46(3), 314-322.
2. Shen, L., Liu, Y. and Xu, H.L. (2010) Treatment of ampicillin-loaded wastewater by combined adsorption and biodegradation. Journal of Chemical Technology & Biotechnology 85(6), 814-820.
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6. Li Y., Liu Y., Shen L., and Chen F. (2008) DO diffusion profile in aerobic granule and its microbiological implications. Enzyme and Microbial Technology, 43, 349-354.

II: Conference Papers

1. Shen, L. and Liu, Y. (2008) Removal of antibiotics by GAC adsorption. Symposium for Young Water Talents, 23 Jun, 2008, Singapore.
2. Liu Y., Li Y., Shen L., Chen F. (2008) Direct measurement of DO diffusion in aerobic granule by a microoxygen electrode. IWA Biofilm Technologies Conference, 111-115, 8-11 Jan, 2008, Singapore.

III: Book Chapters

1. Shen, L., Wang Z. W., Shi F. Q. and Liu, Y. Biosorption isotherms and thermodynamics, in: Liu Y. and Wang J. L. (Eds.) Fundamentals and Applications of Biosorption Isotherms, Kinetics and Thermodynamics, 89-126, Nova Science Publishers, Inc. New York, 2009.
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CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Antibiotics are a type of specially designed pharmaceuticals for control of bacteria in humans and animals. Nowadays, antibiotics have been extensively used as the growth promoter in the livestock industry as well as for clinic therapy. Consequently, the occurrence of antibiotics in wastewaters and other water bodies has been widely reported in many countries, which raises urgent attention of the public. Except for the direct, acute toxic effect for creatures, one of the most serious risks of antibiotics in the water circle is the potential, chronic antimicrobial resistance. This suggests that antibiotics will gradually lose power on the diseases caused by the antibiotic-resistant bacteria. Therefore, the removal of antibiotics from wastewater becomes an emerging challenge for global wastewater industry. Recent scientific efforts have been made to investigate all kinds of treatment technologies for handling the antibiotic-bearing wastewater, ranging from traditional adsorption to advanced oxidation, from physical to biological methods, from single unit to combined system. Currently, relevant information is so limited that is unable to generate a common treatment scheme for the antibiotic-bearing wastewater.

Adsorption by granular activated carbon (GAC) represents a high-efficiency method for removing soluble organic compounds. It should be noted that GAC adsorption capacity can be exhausted when GAC is saturated, meaning that regular regeneration of the exhausted GAC is needed. It appears that adsorption alone is not capable for a long-term, sustainable treatment of antibiotic-bearing wastewater.

Nowadays, biological process is still the core unit of the wastewater treatment plant, which is specifically designed for biological removal of soluble organics and nutrients. However, the conventional biological process is not tailored for treating antibiotics-bearing wastewater due to the specific antimicrobial property of antibiotics.

As compared to the conventional activated sludge process, biofilms have been known to have high resistance to recalcitrant chemicals due to the diffusion limitation that serves as a protective barrier for microorganisms inside the biofilms. In order to overcome the shortcomings of adsorption and biological processes, while to merge their respective advantages, this study attempted to develop a novel combined adsorption-biodegradation system for treating antibiotic-bearing wastewater, in which biofilms developed on GAC and formed the so called biofilm-covered granular activated carbon (BGAC). Although biological activated carbon has been widely used for removal of organic pollutants from various types of wastewaters, including real wastewater from different sources, BGAC has not been reported in treating antibiotics-bearing wastewater. Obviously, the antimicrobial property of this type of wastewater will create more uncertainty and complexity to BGAC.

1.2 OBJECTIVES AND SCOPE

The main objectives of the research are:

(1) To characterize the equilibrium properties of antibiotics adsorption by GAC; to analyze the equilibrium data of antibiotics adsorption with different isotherm equations, further to look into the thermodynamic behaviors of antibiotics

adsorption by GAC. In this study, the most commonly used β-lactam antibiotics (penicillin G, ampicillin and cephalosporin C) were chosen as the model antibiotics.

(2) To investigate the theoretical origins of the empirical first- and second-order rate equations for adsorption, and to verify the proposed theory with the data obtained in this study as well as those from the literature.

(3) To study the biofilm development on GAC in the lab-scale continuous airlift bioreactors; to examine the performance of the biofilm-covered GAC for antibiotic removal in terms of adsorption and biodegradation.

(4) To explore the microbial community of the biofilms developed on GAC for treating antibiotic-bearing wastewater, advanced molecular biology approaches, such as DGGE and 16S rRNA gene sequencing, were employed to investigate the antibiotic-induced changes in the biofilms community diversity, structure and populations.

1.3 ORGANIZATION OF THE THESIS

This thesis consists of six chapters with the following organization:

(1) Chapter 1 is a brief introduction to the research background.

(2) Chapter 2 presents a comprehensive literature review. The review covers the occurrence of antibiotics in different aquatic environments and various technical attempts to treat antibiotics in wastewater treatment plants.

(3) Chapter 3 provides a complete study about the adsorption of three β-lactam antibiotics (i.e. PCG, AMP and CPC) by GAC, including the physical characterization, isotherms, thermodynamics and kinetics. This chapter also explores the theoretical origins of the widely used empirical first- and second-order rate equations for adsorption. It was shown for the first time that first- and second- order rate equations for adsorption are two simplified cases of Langmuir kinetics under certain circumstances. A general simplification principle was further established and confirmed by the adsorption data of antibiotics as well as the literature data.

(4) The focus of Chapter 4 was placed on the biofilms development on GAC in the continuous airlift reactors supplemented with or without AMP. In parallel, for better understanding the removal mechanisms (adsorption and biodegradation) of AMP, a series of batch experiments with BGAC were concurrently carried out in the course of the reactor operation. These allow distinguishing the respective contribution of adsorption and biodegradation to the observed AMP removal.

(5) A detailed study on changes in microbial diversity, structure and population of the biofilm communities with or without exposure to AMP was presented in Chapter 5. Resistant species were found to be predominant in the community of biofilms developed with AMP, and it was also found that the removal of AMP was positively related to the build-up of these functional bacteria in the biofilms.

(6) The major findings of this study and some recommendations for the future investigation are summarized in Chapter 6.

CHAPTER 2

LITERATURE REVIEW

2.1 ANTIBIOTICS

2.1.1 Definition, Classification and Function

Antibiotics are special secondary metabolites derivable from a mold or bacterium that can inhibit or destroy the growth of microorganisms. In 1928, the first antibiotic was discovered by Dr. Fleming and named after the mold culture broth as "penicillin". Afterwards, about 12,000 kinds of antibiotics have been known, among which about 160 kinds have been applied in human clinics. According to their structures, antibiotics can be classified into seven groups (Walsh 2003):

(1) β-lactam antibiotics. The β-lactam antibiotics all contain a β-lactam nucleus in molecular structure. This is a broad class of antibiotics that include penicillin derivatives, cephalosporins, monobactams, carbapenems and β-lactamase inhibitors

(2) Aminoglycoside antibiotics. They are formed by the derivatives of aminohexose, e.g. streptomycin, gentamicin and amikacin.

(3) Polypeptide antibiotics. They have the polypeptide structure, e.g. bacitracin, colistin and polymyxin.

(4) Polyene antibiotics. There are several double bonds in the molecular structure of this group of antibiotics, e.g. nystatin and amphotericin.

(5) Microlide antibiotics. There is a large macrocyclic lactone ring in the molecular structure of this group of antibiotics, e.g. clarithromycin, azithromycin, erythromycin, medecamycin and spiramycin.

(6) Tetracycline antibiotics with four condensed benzene rings, e.g. tetracycline, oxytetracycline and chlortetracycline.

(7) Others, e.g. quinolones, rifampicin, chloramphenicol, sulfonamides and puromycin.

Antibiotics have been used extensively to treat microbial infections in human and animal through the following mechanisms:

(1) Alteration of the formation of the cell wall, e.g. β-lactam antibiotics can inhibit the synthesis of peptidoglycan cross-links in the bacterial cell wall.

(2) Alteration of the function of the cell membrane, e.g. nystatin can bind to ergosterol, and cause holes in the fungi cell membrane.

(3) Inhibition of the synthesis of protein, e.g. aminoglycoside antibiotics.

(4) Inhibition of the synthesis of DNA or RNA, e.g. rifampicin and ciprofloxacin.

2.1.2 Production and Consumption of Antibiotics

Since 1940s, antibiotics have been massively produced through three methods, i.e. microbiological fermentation, partial chemical synthesis and total chemical synthesis. Although most antibiotics may occur in nature, they are normally unavailable in the quantities necessary for the large-scale production. As the total chemical synthesis of antibiotics requires very complex chemical reactions, partial chemical synthesis, called "semisynthesis", is the most efficient method for industrial production of antibiotics. In semisynthesis, fermentation reactions (Figure 2.1) are performed to produce the nucleus-structure compounds (precursors), and subsequently these natural biomolecules are transformed into antibiotics with the desired properties through chemical reactions.

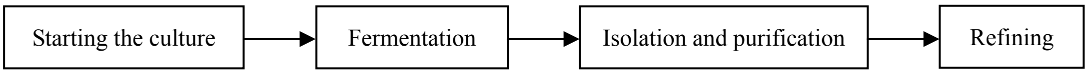


Figure 2.1 Flow diagram of industrial fermentation for antibiotics production.

The market of antibiotic drugs is huge and keeps on growing. In 1996, the world antibiotic market at the level of dosage form was estimated to be US$23 billion. In 2005, worldwide sales of oral antibiotics reached US$25 billion, whereas the global sales of antibiotics increase to US$42 billion in 2009. Among all antibiotics, β-lactam antibiotics account for over 65% of the world antibiotic market. The total world market for β-lactam antibiotics has been estimated to be over US$20 billion (Elander 2003; Hamad 2010). The market of antibiotics continues to grow at a significant rate, with adult and pediatric prescriptions growing by 8.5% and 10.8%, respectively, in 2005 (Christoffersen 2006). Specifically, sales of penicillin class drugs have increased 5% from 2005 to 2009 (Hamad 2010). Another major use of antibiotics is to enhance the growth of livestock. For example, consumption of antibiotics in 1997 in Denmark exceeded more than 150,000 kg, out of which more than 100,000 kg were used as growth promoters (Jensen 2001). In USA, usage of antibiotics as growth promoter has been estimated about 24.6 million pounds a year （70% of all antibiotics consumed) in the past decades (Lilliston 2010). Similar trend has been observed in many other countries, e.g. China, Australia, New Zealand, and EU (Sarmah et al. 2006; Zhao et al. 2010).

2.2 SIGNIFICANCE OF ANTIBIOTICS IN THE ENVIRONMENT

2.2.1 Occurrence of Antibiotics in the Environment

As discussed above, a large amount of antibiotics has been used for various purposes. After taken by the end users (human being or animal) as anti-infection drugs or growth promoters, some antibiotics may be significantly metabolized, whereas many are intact and thereafter excreted out with urine and feces. Moreover, substantial amounts of unused antibiotics have been thrown away by consumers all over the world. No matter disposed in the sewer directly, or through leaching and overland run-off, antibiotics will finally enter the aquatic environment and recycle in the biosphere. Figure 2.2 shows the anticipated occurrence compartments and the exposure pathways of antibiotics in the environment. However, the public is alerted to the spread of these substances in the environment only recently, as antibiotics in the environment is of trace amount and hard to detect. Nowadays, with the development of the more sophisticated analytical methods (e.g. solid phase extraction (SPE), high-performance liquid chromatography (HPLC) and liquid chromatograph-mass spectrometer-mass spectrometer (LC-MS-MS)), numerous kinds of antibiotics have been detected in different environmental compartments (Seifrtová et al. 2009).

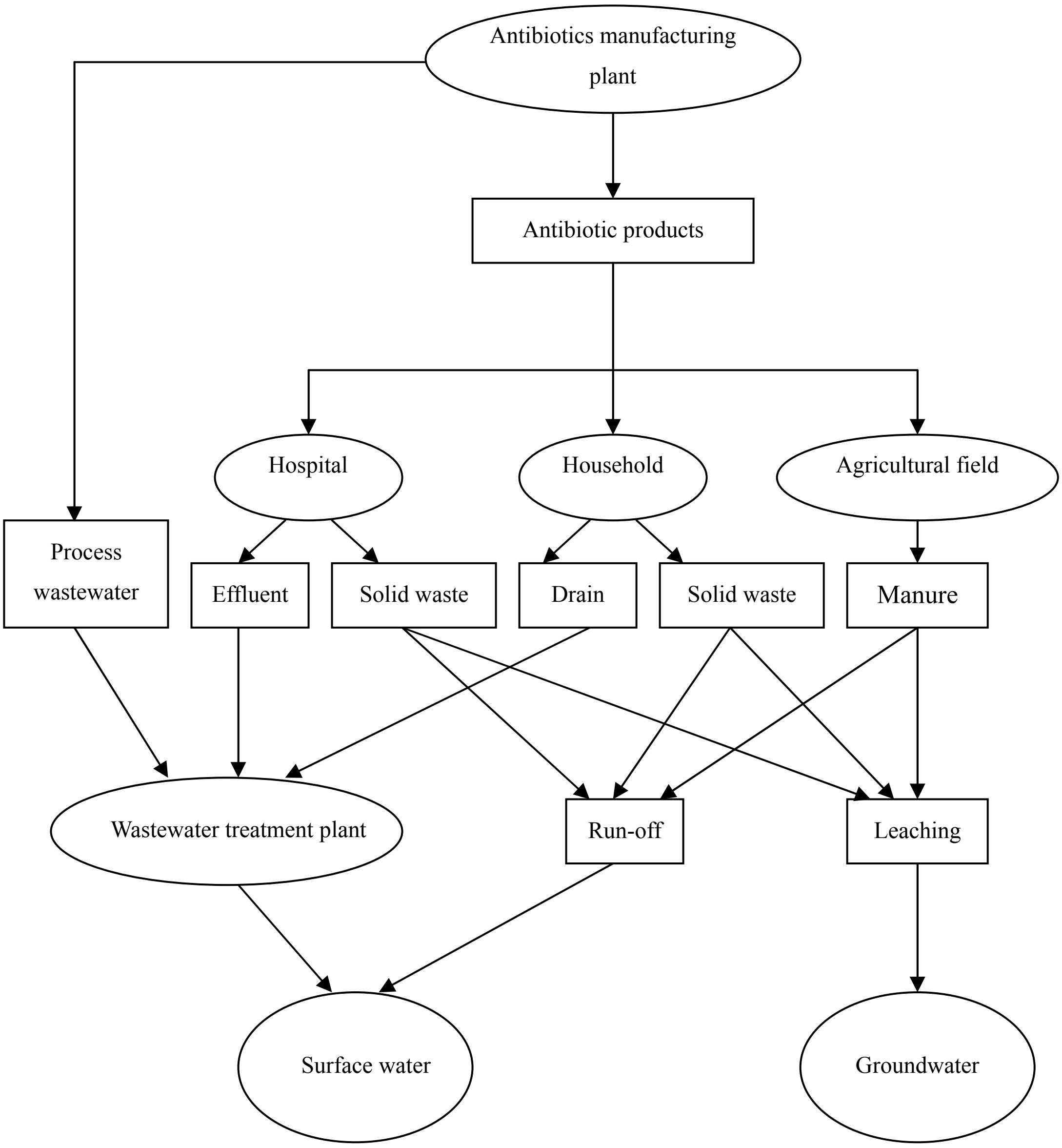


Figure 2.2 Occurrence and pathways of antibiotics in the environment.

Manufacture Wastewater

China is the largest producer of antibiotics in the world, with an annual production of 11,000 metric tons, which is about half of the global production (Bergeron 2004). According to Yang et al. (1997), the discharge of pharmaceutical wastewater from the antibiotics manufacturing process is responsible for antibiotics spread in the water cycle. Manufacture wastewater often has (1) high chemical oxygen demand (COD) concentration (10,000 to 80,000 mg L^{-1}) and high suspended solid concentration (500 to 25,000 mg L^{-1}); (2) high toxicity due to high-strength antibiotic residues, sulphate, surfactant, acid, base, organic solvent, etc.; (3) low pH, high temperature, strong color and smell; (4) large variation in the quantity and quality, and (5) a large quantity of wastewater produced (e.g. 150 to 850 m^3 ton^{-1} product). More details are presented in Table 2.1.

Table 2.1 Characteristics of several typical antibiotics manufacture wastewater (unit: mg L^{-1}) (Yang et al. 1997).

Product Name	Production Process	COD	SS	SO_4^{2-}	Antibiotic Residue
Penicillin	Extraction	15000-80000	5000-23000	5000	
Ampicillin	solvent recovery	5000-70000		<50	0.54% (open ring)
Streptomycin	Extraction	10000-16000	1000-2000	2000-5500	
Kanamycin	Extraction	25000-30000	<250		80
Gentamicin	Extraction	25000-40000	10000-25000	4000	50-70
Tetracycline	crystalization	20000			1500
Oxyletracycoine	crystalization	10000-35000	2000	2000	500-1000
Medecamycin	crystalization	15000-40000	1000	4000	760
Lincomycin	solvent recovery	15000-20000	1000	<1000	50-100
Choltetracycline	crystalization	25000-30000	1000-5000		80

India is another major producer of antibiotics in the world market. The effluents from some Indian drug plants were found to have extremely high levels of antibiotic residues, e.g. up to 31 mg L^{-1} for ciprofloxacin (Larsson et al. 2007).

Hospital Wastewater

Hospital wastewater is another main source for the occurrence of antibiotics in the environment. The share of antibiotic drugs in hospital varies from a low percentage (e.g. 13% for clarithromycin, 7% for erythromycin and 4% for roxithromycin) to over 80% (e.g. 86% for ampicillin and 94% for penicillin G). These may lead to a complex hospital wastewater with residues of various antibiotics (Ternes and Joss

2006). In Germany, the total emission of antibiotics from hospitals and households resulted in a high antibiotic residual concentration of up to 71 mg L^{-1} in the municipal sewage, half of which was attributable to penicillins (Kümmerer and Henninger 2003). Typical antibiotics detected in a wide variety of hospital wastewaters are shown in Table 2.2.

Table 2.2 Antibiotics detected in hospital wastewater.

No.	Antibiotics /Concentration (μg L^{-1})	Country	Reference
1	Sulfamethoxazole/0.8; Trimethoprim/5; Ofloxacin/25.5; Ciprofloxacin/2; Lincomycin/2	USA	Brown et al. 2006
2	Sulfamethoxazole/2.1; Trimethoprim/2.9; Ofloxacin/34.5; Lincomycin/0.3; Penicillin G/5.2	USA	Brown et al. 2006
3	Sulfamethoxazole/0.4; Ofloxacin/35.5; Ciprofloxacin/0.85; Penicillin G/0.85	USA	Brown et al. 2006
4	Ampicillin/20-80	Germany	Kümmerer and Henninger 2003
5	Ciprofloxacin/0.7-124.5	Germany	Hartmann et al. 1999
6	Ciprofloxacin/17-30; Norfloxacin/2.6-7	Swiss	Kümmerer 2004
7	Ciprofloxacin/3.6-101.0; Metronidazole/0.1-90.2; Sulfamethoxazole/0.4-12.8; Ofloxacin/0.2-7.6; Trimethoprim/0.6-7.6; Doxycycline/0.6-6.7	Sweden	Lindberg et al. 2004

Wastewater Treatment Plants (WWTPs)

Manufacture and hospital wastewaters containing antibiotics are eventually diluted when reaching the municipal wastewater treatment plants (WWTPs). In Germany, Hirsch et al. (1999) first investigated the occurrence of antibiotics in WWTPs, and found 18 antibiotic substances in the classes of macrolides, sulfonamides, penicillins, and tetracyclines, while the frequent occurrence of erythromycin-H_2O, roxithromycin and sulfamethoxazole was detected with concentrations of up to 6 μg L^{-1} in the WWTPs. A recent study revealed that very high concentration of ciprofloxacin (14 mg L^{-1}) was found in the effluent of a WWTP receiving wastewater from drug manufacturers in India (Fick et al. 2009). Table 2.3 shows the antibiotic residues detected in WWTPs in different countries.

Table 2.3 Antibiotics detected in wastewater treatment plant.

No.	Antibiotics /Concentration (μg L^{-1})	Country	Reference
1	Sulfamethazine/0.21; Sulfamethoxazole/1.25; Tetracycline/1.2; Ciprofloxacin/0.21; Erythromycin-H_2O/1.2; Trimethoprim/1.3	USA	Karthikeyan and Meyer 2006
2	Ciprofloxacin /0.20-1.4; Sulfamethoxazole/0.21-2.8; Tetracycline/0.061-1.1; Trimethoprim /0.21-7.9 for	USA	Batt et al. 2007
3	Clarithromycin/0.087; Erythromycin-H2O/0.080; Ciprofloxacin/0.118; Ofloxacin/0.094; Sulfamethazine/0.363; Sulfamethoxazole/0.243;Tetracycline/0.151; Doxycycline/0.038	Canada	Miao et al. 2004
4	Sulfamethoxazole/0.6	Spain	Carballa et al. 2005
5	Clarithromycin/0.057-0.33	Switzerland	McArdell et al. 2003
6	Sulfamethoxazole/0.674; Trimethoprim/1.3; Doxycycline/2.48	Sweden	Lindberg et al. 2005
7	Sulfamethoxazole/0.13; Erythromycin/0.13	Korea	Kim et al. 2007
8	Ciprofloxacin/45.0; Sulfamethoxazole/0.688; Trimethoprim/0.425; Lincomycin/0.315	Korea	Sim et al. 2010
9	Ofloxacin/0.165; Norfloxacin/0.085; Roxithromycin/0.278; Erythromycin-H_2O/2.054; Sulfamethoxazole/0.078	China	Xu et al. 2007
10	Levofloxacin/0.301; Clarithromycin/0.359; Azithromycin/0.138	Japan	Yasojima et al. 2006

Surface Water

The presence of antibiotics in the surface water has been widely reported. Table 2.4 shows the occurrence of antibiotics in different kinds of surface waters around the world.

Table 2.4 Antibiotics detected in surface water.

No.	Antibiotics /Concentration (ng L^{-1})	Site	Reference
1	Sulfacetamide/2.1-4.4; Sulfadiazine/5.7-12.9; Sulfamethoxazole/3.6-13.0;Sulfadimethoxine/ 2.2; Phthalylsulfathiazole /9.2-32.2; Sulfamethylthiazole/5.3-6.2; Sulfachloropyridazine/2.7-2.9	Bohai Sea estuary, China	Ye et al. 2007
2	Erythromycin /1.8-4.8; Sulfamethoxazole/1.7-36	Han River, Nakdong River and Youngsan River, Korea	Kim et al. 2007
3	Sulfapyridine/121; Sulfadiazine/236; Sulfamethoxazole/402; Sulfadimethoxine/28	Tevere river (proximal to hospital), Italy	Perret et al. 2006
4	Sulfadiazine/66; Sulfadimethoxine/74	Trigno river (proximal to cattle-breeding), Italy	Perret et al. 2006
5	Erythromycin-H_2O/1700	From creek Urselbach to river Rhine, Germany	Hirsch et al. 1999
6	Clarithromycin/75	Glatt river, Switzerland	Giger et al. 2003
7	Chlortetacycline/150; Tetracycline/110	Snake Creek, USA	Lindsey et al. 2001
8	Sulfadimethoxine/60; sulfamerazine/220	North Dry Creek, USA	Lindsey et al. 2001
9	Sulfamethoxazole/1020	Cuyahoga River, USA	Lindsey et al. 2001
10	Ciprofloxacin/20; Chlortetracycline/420; Lincomycin/60; Norfloxacin/120; etc	139 stream sites, USA	Kolpin et al. 2002
11	Trimethoprim/660 ; Enalapril/1500	Isakavagu-Nakkavagu River (proximal to drug manufacture), India	Fick et al. 2009

It can be seen that high concentrations of antibiotics occur at sites close to antibiotics application hubs, e.g. hospital or animal farm. So far, the spatial and temporal distributions of antibiotics in rivers and other surface waters are still under investigation.

Generally, β-lactam antibiotics were thought not to occur in the surface water for the poor stability of the β-lactam ring. However, recent studies showed that this group of antibiotics still can be detected in the natural aquifers due to the extremely wide use of β-lactam antibiotics. For example, Christian et al. (2003) reported that the concentrations of ampicillin, amoxicillin, mezlocillin, flucloxacillin and piperacillin in surface water could be as high as 48 ng L^{-1}. In China, five β-lactam antibiotics (penicillin G, amoxicillin, ampicillin, nafcillin and mezlocillin) were found at concentrations up to 9.56 μg L^{-1} in the river water at the downstream of antibiotics manufacturers (Yin et al. 2010).

Groundwater

The occurrence of antibiotics in the groundwater is related to the antibiotic residues in the soil. For example, Holm et al. (1995) found a variety of sulfonamides at concentrations of up to 5 mg L^{-1} in the groundwater under a landfill in Grinsted (Denmark), which was used for the disposal of pharmaceutical production wastes. Through analysis of a large number of groundwater samples in Germany, Hirsch et al. (1999) found that four samples collected from an agricultural area contained sulfonamide antibiotics at concentrations of above 0.02 μg L^{-1}, and two samples taken from a sewage irrigation field contained 0.08 to 0.16 μg L^{-1} of sulfamethoxazole. In addition, tetracyclines at 11 to 540 mg L^{-1}, β-lactam antibiotics at 2.1 to 3.5 mg L^{-1} and macrolides at 15 to 275 mg L^{-1}, were found in lagoon wastewater and agricultural drainage wells (APHA et al. 2000). The similar situations were found worldwide.

Drinking Water

It appears from the above discussion that antibiotics are extensively distributed in the aquatic environment, and the occurrence of antibiotics in drinking water has accordingly raised special safety concerns. Various antibiotics have been detected in drinking water samples. For example, in a monitoring program in drinking water facilities across Germany, trace amounts of sulfamethoxazole were detected in the range of 13 to 45 $\mu g\ L^{-1}$ in 6 out of 51 samples (Muckter et al. 2004).

2.2.2 Resistance of Antibiotics

Definition of Antibiotic Resistance

Antibiotic resistance is the ability of a microorganism to withstand the effects of an antibiotic. Antibiotic resistance is usually measured as the minimum concentration required for achieving a definable effect (e.g. growth inhibition) on the microbial cells. Wherever there is a change in susceptibility that renders an agent ineffective against a certain microorganism, this microorganism is referred to be resistant. Many microorganisms are insensitive and thereby intrinsically resistant to a particular agent by their physiology or biochemistry nature. Susceptible microorganisms can become insensitive by mutation or by incorporation of the genetic information which encodes the resistance (Kummerer 2004).

Antibiotic resistance evolves naturally via natural selection through random mutation, but it could also be engineered by applying an evolutionary stress on a population. Once such a gene is generated, bacteria can transfer the genetic information in a horizontal fashion (between individuals) by plasmid exchange. If a bacterium carries several resistance genes, it is called multiresistant, or informally, a superbug. The outbreaks of resistance can be geographically scattered to all the antibiotic groups, suggesting that antibiotic resistance could spread fast (Walsh

2003).

Mechanisms of Antibiotic Resistance

Historically, a successful antibiotic is targeted on some single, essential bacterial functions, typically carried out by a chromosomally encoded protein. A mutation in the DNA that codes for this protein will render the protein slightly impaired in its normal function, which in turn makes the mutant bacterium resistant to that antibiotic. As shown in Figure 2.3, several mechanisms of antibiotic resistance have evolved in bacteria, including:

(1) Enzymatic destruction or modification of the antibiotic, which is also the most common mode;

(2) Alteration of the antibiotic target site;

(3) The active export or so called "efflux pumps" of antibiotics.

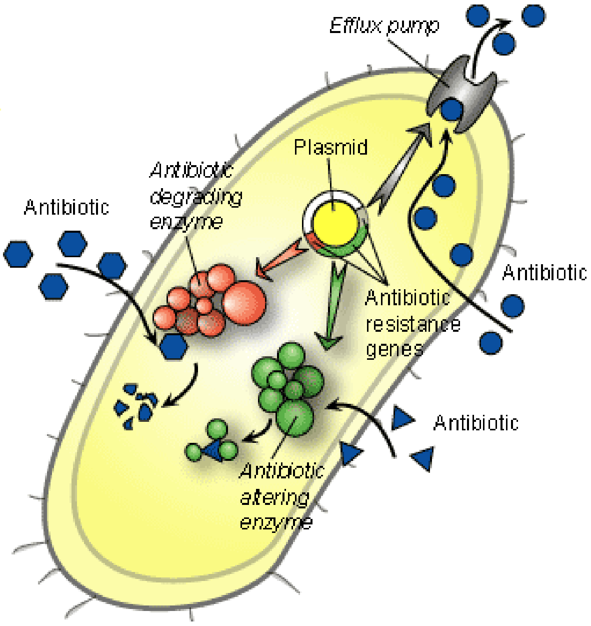


Figure 2.3 Mechanisms of antibiotic resistance (Yim 2006).

For β-lactam antibiotics, the most widespread mode of resistance is the first strategy, specifically the expression of β-lactamases that hydrolyze the four-membered β-lactam ring in both penicillin and cephalosporin classes of antibiotics (Figure 2.4).

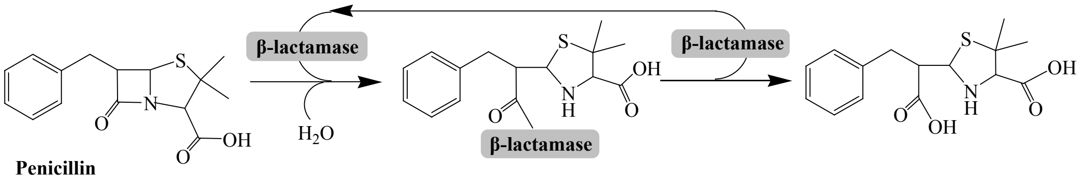
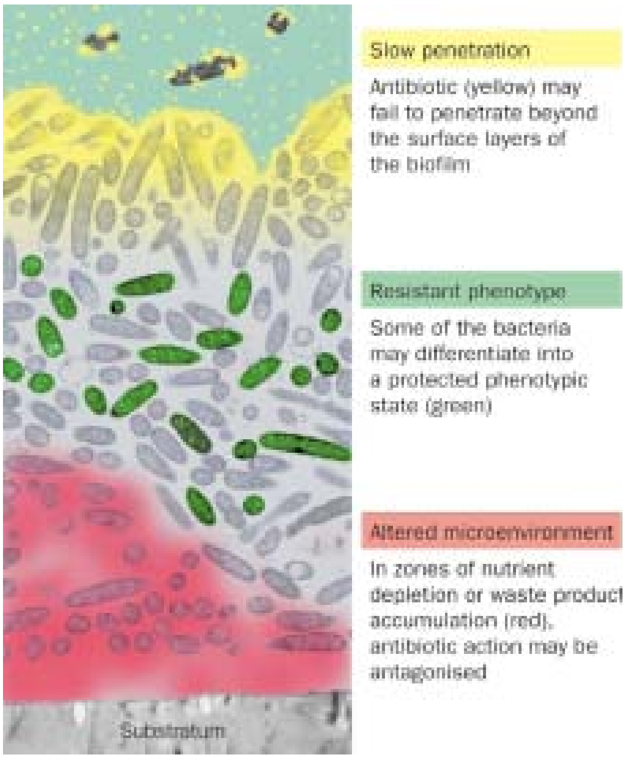


Figure 2.4 Hydrolytic ring opening and deactivation of penicillin by β-lactamase (Walsh 2003).



Slow penetration

Antibiotic (yellow) may fail to penetrate beyond the surface layers of the biofilm

Resistant phenotype

Some of the bacteria may differentiate into a protected phenotypic state (green)

Altered microenvironment

In zones of nutrient depletion or waste product accumulation (red), antibiotic action may be antagonised

Figure 2.5 Three hypotheses for mechanisms of antibiotic resistance in biofilms (Stewart and Costerton 2001).

Note: The attachment surface is shown at the bottom and the aqueous phase containing the antibiotic at the top.

When bacteria adhere to some media and encase themselves in a matrix of polysaccharides and proteins, a layer known as “biofilm” will be formed. Preliminary evidence indicates that the conventional antibiotic resistance mechanisms are not sufficient to explain most cases of antibiotic resistance in biofilms. Three main hypotheses for the mechanisms of resistance to antibiotics in bacterial biofilms are elucidated in Figure 2.5.

Horizontal Gene Transfer (HGT)

As discussed above, the mutant bacteria may survive well in the antibiotic present environment. Meanwhile, the non-mutant bacteria are better to compete for resources and reproduce faster than the mutant form. When antibiotic is absent, resistant bacterial cells will be at a disadvantage in the wild, and then not persist or spread. This means that in an environment exposed to antibiotics periodically rather than continuously, it is unlikely that antibiotic resistance would become an epidemic problem if the single chromosomal mutation is its sole genetic basis. However, vertical inheritance of single resistance mutations does take place. Based on laboratory studies of bacteria in pure culture, it is well-known that changes in the DNA sequence of the bacterial cell can endow that cell with a new property, and all subsequent progeny will inherit that property because bacteria are haploid. So far, it is an enormously incomplete picture of the genetic basis of antibiotic resistance in the real world. Genes encoding antibiotic resistance may transfer from some bacterial cells to others, even from non-pathogenic bacteria to pathogenic ones. This transferring of genetic information intercellular among bacteria is called horizontal gene transfer (HGT) (Summers 2006). Transformation, transduction, and conjugation are three known ways for HGT as illustrated in Figure 2.6. Only a few bacteria are able to use all of these ways, whereas many free living bacteria appear to use at least one of these means to share genetic information horizontally.

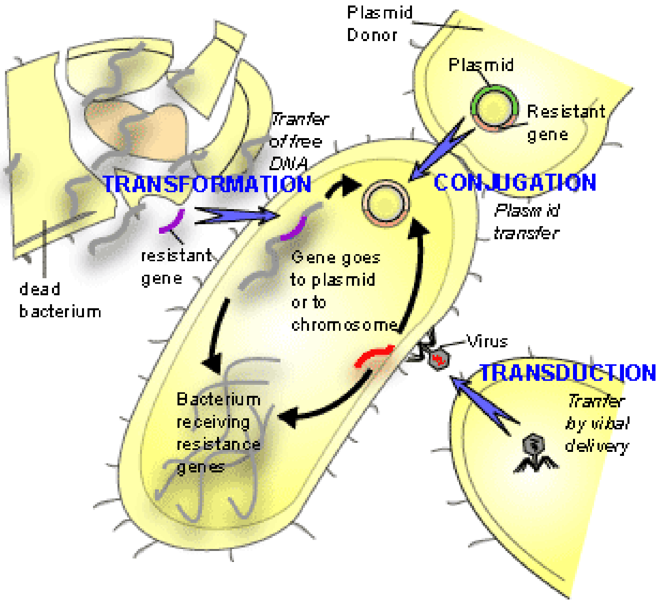


Figure 2.6 Horizontal gene transfer (Yim 2006).

Resistant Bacteria in the Environment

Since antibiotic resistance may quickly spread out from resistant bacteria to normal bacteria through HGT, special attentions should be given to the antibiotic resistant bacteria in the environment. Some common bacteria in the environment and their possible resistance are listed below.

(1) *Escherichia coli*

Resistant *Escherichia coli* strains have been found in the hospital effluent, sewage and surface water. In Korea, coliform bacteria isolated from the aquatic environment were reported to be highly resistant to sulfamethoxazole, aminoglycosides, and β-lactam antibiotics (Park et al. 2003). Reinthaler et al. (2003) investigated the discharge from both hospital and WWTPs in Austria, and found *E. coli* strains in the sewage were resistant to 16 out of 24 tested antibiotics. Iwane et al. (2001) also found that some *E. coli* and coliform group bacteria in the Tama River (Japan), as well as a wastewater treatment plant located on the river, were resistant to seven antibiotics.

(2) *Staphylococcus aureus*

S. aureus is the first bug reported to battle penicillin. This bacterium is often a harmless passenger in the human body, but it can cause diseases, such as pneumonia and toxic shock syndrome, when it overgrows or produces a toxin. *S. aureus*, a catalase-positive, gram-positive and cocci-shaped bacterium, has been implicated in hospital acquired infections since 1950 when the microorganism developed a resistant to penicillin. Even during the golden age of antibiotics, about 50% of *S. aureus* strains were found to be resistant to penicillin and later penicillin derivative drugs. The microorganism quickly becomes resistant to newer and more powerful antibiotics, such as tetracycline and the aminoglycosides. *S. aureus* is a member of the Micrococcaceae family which comprises four genera: Micrococcus, Staphylococcus, Planococcus, and Stomatococcus. Staphyolococcus are natural habitants of skin and mucouse membranes of humans. These bacteria can be easily found in hospitals. Most species of Staphycococcus are opportunistic pathogens, *S. aureus*, however, has been considered a serious bacterial pathogen since the organism developed a resistance to penicillin in the 1950s.

(3) *Streptococcus pneumoniae*

S. pneumoniae, one of the normal flora found in the upper respiratory tract and especially in the oropharynx of children, is the leading cause of community-acquired pneumonia, meningitis and otitis worldwide. Harakeh et al. (2006) isolated and molecularly characterized *S. pneumoniae* in samples collected from the aquatic environment in Lebanon. After testing the resistance of the 20 isolates to various antibiotics, Harakeh et al. (2006) found that all the isolates were resistant to vancomycin, gentamicin, and oxacilli, whereas 62.5% of the isolates were resistant to clindamycin, and only 25% were erythromycin resistant.

2.2.3 Impact of Antibiotics on the Ecosystem

2.2.3.1 Acute and chronic toxicity

The ecological risk of antibiotics is usually estimated by their measured environmental concentrations and predicted no-effect concentrations (Lindberg et al. 2007). Park and Choi (2008) evaluated the acute and chronic aquatic toxicities of eleven commonly used antibiotics including β-lactams based on the above two concnetartion criteria. It was found that the acute toxicity of β-lactam antibiotics was weak. However, the potential chronic ecological implication of these antibiotics should be further studied.

2.2.3.2 Alteration of the microbial community

Recent studies showed how bacterial species would respond to the antibiotics entering the natural environment. Munoz-Aguayo et al. (2007) investigated the selection potential of chlortetracyclin (CTC) for resistance in aerobic bacterial populations in river water, and found that the CTC-resistant bacteria were increased with the increase of the CTC concentration. Zielezny et al. (2006) looked into the bacterial community structure in the soil with impact of sulfadiazine (SDZ). Results showed a pronounced difference between the soil sample supplemented with 10 mg kg^{-1} SDZ and control free of SDZ. To date, most studies about the potential impact of antibiotic residues on the microbial community are related to soils (Hammesfahr et al. 2008; Zielezny et al. 2006), whereas few reports focus on bacterial community in the aquatic environment (Costanzo et al. 2005) and marine sediments (Naslund et al. 2008). It should be pointed out that these studies are limited to the natural environments, whereas did not cover the biological systems in WWTPs. This knowledge gap should be made up in the future study.

2.2.3.3 Molecular biology approaches for community analysis

The ecosystems, including WWTPs, contained a tremendous amount of various species, most of which are viable, but non-culturable (Grady et al. 1999). This poses a large difficulty to explore the microbial community. Fortunately, there are remarkable progresses in molecular microbiology in recent years, which provide useful tools for study of microbial community at the genetic level. 16S rRNA gene sequence analysis is one of the molecular biology techniques that can be used in bacterial classification. The application of the 16S rRNA gene as a molecular chronometer has been reviewed extensively by Woese (1987). This procedure has become a standard practice in bacteriology, i.e. determination of the 16S rRNA gene sequence is a preceding step for characterization of an isolate (Clarridge 2004). 16S rRNA gene sequencing has also become very popular in the area of environmental microbiology. Non-culturable microbes in the mixture can be investigated through cloning and sequencing 16S ribosomal RNA (rRNA) encoding genes, as well as culturable microorganisms (Hugenholtz et al. 1998). Moreover, genetic fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE) supply an efficient tool to observe the diversity of various microbial communities in different environments (Muyzer 1999). DGGE was originally developed to analyze fragments from single organisms (Fischer and Lerman 1983). Muyzer et al. (1993) first used this technique to analyze complex mixtures of microorganisms and proved that it was feasible to study microbial ecology. In DGGE, DNA fragments of the same length but with a different base-pair sequences can be separated according to melting properties, and theoretically a band represents a genus or specie of microbe (Yang et al. 2009).

Numerical taxonomy has also been developed from macro scale ecology to the microorganism community. Basically, a community can be characterized by the diversity within itself and the similarity with each other. For this, many different indices have been proposed to represent temporal diversity magnitude (Stocker et al. 1985). Each of these indices has strengths and weaknesses, thus it is suggested to use a combination of several indices to take advantages of each index and develop a more complete understanding of community structure. In parallel, coefficients can be calculated between two communities and a similarity matrix can be constructed for comparison. Similarity matrices can be graphically displayed as a dendrogram (Fromin et al. 2002). Moreover, it should be noted that the development of computer technology further benefits the study of microbial community. Software, such as Quantity One (Bio-Rad Laboratories, Hercules, USA), GelCompar II (Applied Maths, Kortrijk, Belgium) and Bionumerics (Applied Maths, Kortrijk, Belgium), have been developed as powerful tools for statistical interpretation of electrophoretic images.

2.3 REMOVAL OF ANTIBIOTICS FROM WASTEWATERS

In general, wastewater and water treatment can be divided into physical, chemical and biological methods or their combination. As shown above, antibiotics have antimicrobial structures and properties, thus treatment of antibiotics appears to be a very challenging issue. Therefore, new technologies as well as conventional technologies for removing antibiotics from wastewaters are reviewed.

2.3.1 Adsorption

Adsorption is defined as the inter phase accumulation or concentration of substances at a surface or interface (Weber 1972). Adsorption plays an important role in refurbishing the water quality. In fact, purification, decolorization and detoxification of water have been practiced for centuries using charcoals and activated carbons. Activated carbon has a high internal surface area created from pores of various sizes, i.e. macropores with 'diameters' in excess of 50 nm, mesopores with 'diameters' in the range 2 to 50 nm, and micropores with 'diameters' smaller than 2nm (Thomson and Crittenden 1998). This porous structure makes activated carbon accessible to the components being removed from liquid. Recently, application of adsorption process has been extended for removing antibiotics from wastewater.

As a widely used adsorbent, activated carbon has been applied for the removal of antibiotics. For example, powered activated carbon (PAC) has been used for removing the sulphonamide class of antibiotics with efficiency of over 90% at typical plant dosages (Adams et al. 2002), and granular activated carbon (GAC) can adsorb trimethoprim with efficiency up to 90% (Kim et al. 2010). Moreover, Rivera-Utrilla et al. (2009) studied the adsorption of nitroimidazole antibiotics by three kinds of activated carbon with particle size of 0.45 to 1 mm. It was shown that the π-π dispersion type interactions between carbon graphene layers and nitroimidazole aromatic rings were responsible for the adsorption of nitroimidazole antibiotics by the activated carbons, meanwhile the pH of the medium and the concentration of the electrolyte present were not the major factors in that case.

Other adsorbent materials have also been used to remove antibiotics from aqueous solution. Goyne et al. (2005) used mesoporous and nonporous SiO_2 and Al_2O_3 as adsorbents to react with the antibiotic ofloxacin. It was found that the zwitterionic compound was adsorbed to SiO_2 solids through the protonated N_4 in the piperazinyl group and, possibly, a cation bridge; whereas the antibiotic adsorbed to Al_2O_3 solids through the ketone and carboxylate functional groups via a ligand exchange mechanism. Otker et al. (2005) investigated the adsorption of enrofloxacin, a fluoroquinolone group antibiotic, on natural zeolite, and found that the process was highly pH dependent. Similarly, Dutta et al. (1999) also studied the adsorption of 7-aminocephalosporanic acid, cephalexin, cefadroxyl and 6-aminopenillanic acid by four types of polymeric resins as well as activated carbon.

2.3.2 Biological Processes

2.3.2.1 Biodegradability of antibiotics

Antibiotics have an adverse effect on microorganisms, thus it is imperative to evaluate the impact of these substances on the bacterial activity. The activity of aerobic microorganisms can be measured by the amount of oxygen consumed or the amount of carbon dioxide produced, whereas the activity of anaerobic microorganism is determined by the amount of methane produced. For such purpose, closed bottle test (CBT) is recommended as a fast, simple mean for the assessment of the biodegradability of organic compounds (Nyholm 1991; OECD 1992). In accordance with the OECD guidelines, if biodegradability expressed as a percentage of oxygen consumed in the testing vessel, exceeds 60% of the theoretical oxygen demand in 28 days, the tested substance can be classified as readily biodegradable. In addition, a toxicity control should be performed in the CBT and the colony forming units (CFUs) be monitored in order to obtain further information about the effects of the tested antibiotics on the bacteria present in the testing vessel. This technique has been employed to investigate the biodegradability of many clinic antibiotics and representative antibiotics in wastewaters. For example, Al-Ahmad et al. (1999) found that the biodegradability of penicillin G was 36% after 40 days of CBT culture. Ingerslev et al. (2000) also reported that for 12 different sulfonamides tested, none of them were readily biodegradable. Similarly, Alexy et al. (2004) found that 18 clinically important antibiotics studied were all non-readily biodegradable. Generally, non- readily biodegradable substances are supposed not to be effectively removed by biological treatment. It should be pointed out that biodegradability of chemical compounds determined by the CBT method has been questioned for decades. This is due to the fact that the CBT culture could not reasonably reflect the situation and conditions in the real WWTPs. Thus, one should be prudent when interpreting and using the CBT data for the process design and control.

2.3.2.2 Anaerobic treatment of antibiotic wastewater

Anaerobic treatment is a multi-step biochemical process including hydrolysis, acidogenesis and methanogenesis. In the hydrolysis phase, extracellular enzymes break down solid complex organics, cellulose, proteins, lignins, and lipids into soluble organic fatty acids, alcohols, carbon dioxide, and ammonia, while microorganisms convert the products of the first stage into acetic acid, propionic acid, hydrogen, carbon dioxide, and other low molecular weight organic acids in the acidogenesis phase. In the last stage, i.e. the methanogenesis phase, two groups of methane-forming bacteria work concurrently to converting hydrogen and carbon dioxide to methane, whereas acetate to methane and bicarbonate, respectively. Anaerobic process has been widely applied to remove high-strength organic compounds as well as to decompose refractory substances. Rodriguez-Martinez et al. (2005) investigated the anaerobic treatment of penicillin G production wastewater with an organic loading rate up to 2.09 g COD L^{-1} day^{-1}, in an upflow anaerobic sludge blanket reactor. It was found that the removal efficiency for COD and sulfate were both about 90%, which demonstrates the potential of an anaerobic treatment of antibiotic manufacture wastewater containing a high COD level along with sulfates, nitrate, methylene chloride, etc.. Some other examples of anaerobic treatment of antibiotic production wastewater are summarized in Table 2.5.

Table 2.5 Anaerobic treatment of antibiotic wastewater.

No.	Influent COD ($mg L^{-1}$)	Influent antibiotics ($mg L^{-1}$)	COD removal efficiency (%)	Antibiotics removal efficiency (%)	Reactor	Reference
1	9736-19862	Ampicillin 3.2; aureomycin 1.0	91.0	Ampicillin 67.8; aureomycin 51.5	Anaerobic baffled reactor	Zhou et al. 2006
2	7000±800	Tylosin 20-200	70-75	95	Upflow anaerobic stage reactor	Chelliapan et al. 2006
3	25700	Penicillin	57.8-69.1	N/A	Anaerobic fixed film fixed bed reactor	Satyanarayan et al. 2003
4	3000-43000	Penicillin	97	N/A	Stirred reactor	Degirmentas and Deveci 2004
5	12000-15000	Cephalexin	88.5	N/A	Anaerobic fluidized bed reactor	Saravanane et al. 2001
6	127000	Erythromycin 1-200	94.7	N/A	Anaerobic sequencing batch reactor	Amin et al. 2006
7	3000	Sulfamerazine 10-90	89-68	76-100	Upflow anaerobic sludge blanket reactor	Sponza and Demirden 2007

2.3.2.3 Aerobic treatment of antibiotic wastewater

In aerobic process, organic compounds can be completely decomposed to carbon dioxide (Metcalf & Eddy 2004). Examples of aerobic treatment of antibiotic-bearing wastewater are given in Table 2.6.

Table 2.6 Aerobic treatment of antibiotic wastewater.

No.	Influent COD (mg L^{-1})	Influent antibiotics (mg L^{-1})	COD removal efficiency (%)	Antibiotics removal efficiency (%)	Reactor	Reference
1	998-1662	Ampicillin 0.6; aureomycin 2.3	73.1-89.2	Ampicillin <10; aureomycin <10	Biofilm airlift suspension reactor	Zhou et al. 2006
2	330-1080	Sulfamerazine	71-85	98-100	Continuously stirred tank reactor	Sponza and Demirden 2007
3	N/A	Sulfamethoxazole 20-383	N/A	100	Sequencing batch reactor	Drillia et al. 2005
4	N/A	Tetracycline 0.0001-0.0006	N/A	78.4-86.4	Sequencing batch reactor	Kim et al. 2005

It appears from Tables 2.5 and 2.6 that anaerobic process has been more frequently applied than aerobic process for antibiotic wastewater treatment. Nevertheless, Table 2.6 shows that some antibiotics still can be completely degraded to carbon dioxide and water under aerobic conditions. For high-strength wastewater from antibiotics manufacturers, aerobic process would not be viable and dilution of wastewater is needed before aerobic treatment. Therefore, the integrated anaerobic-aerobic process or the micro-aerobic process has been reported for treating high-strength antibiotic manufacture wastewater (Qi et al. 2005; Zhou et al. 2006; Sponza and Demirden 2007). In the most studies discussed above, COD has often been used as an indicator of the process performance. However, it should be pointed out that high COD removal efficiency does not mean a high antibiotic removal. Hence, in the future study, direct determination of antibiotic concentration instead of COD is strongly recommended.

2.3.2.4 Biofilm technology

Generally, microorganisms applied in the biological process are in two forms, i.e. suspended activated sludge and biofilms. Biofilms can be defined as the aggregates of microorganisms growing on a solid packing material, namely carrier (Metcalf & Eddy 2004). So far, biofilms have been recognized as highly complex, heterogeneous and uncontrollable structures. The observed biofilm thickness and morphology would result from the operation conditions of the biofilm system (Van Loosdrecht et al. 1995). Generally, biofilms can be described as a mushroom-like cluster consisting of holes or voids. Voids are open channels that are connected to the interior of biofilms with the bulk fluid. de Beer and Stoodley (1995) found that liquid could flow through the voids, but was always stagnant in the cell clusters. Consequently, both diffusion and convection may contribute to mass transfer in voids, while transport is determined by diffusion only in cell clusters. Compared to suspended sludge, biofilms have the following advantages for treating recalcitrant organic pollutants including antibiotics (Nicolella et al. 2000; Wilderer and McSwain 2004):

(1) Biofilms have very long biomass retention time, which makes them particularly suitable when treatment requires slow growing microorganisms with poor biomass yield or when the wastewater contains toxic components for growth of microorganisms.

(2) Microorganisms growing in the biofilm community also receive protection from the extracellular polymeric substances (EPS) in which they are embedded. EPS can minimize the impact of changes in pH, temperature, and concentration of toxic substances.

(3) Non-readily biodegradable matter can be adsorbed onto and transferred into the biofilm and periodically removed by desorption and subsequent biodegradation.

As noted by Jones et al. (2005), microorganisms may gradually become acclimatized to certain chemicals and therefore may degrade them more effectively. This indicates that biofilms should be more beneficial for the functional bacteria that are able to biodegrade the recalcitrant pollutants. Zwiener et al. (2003) investigated the biological treatment of a non-antibiotic pharmaceutical ibuprofen with activated sludge and biofilms, respectively. It was found that the oxic biofilm reactor had a higher degree of biodegradation for ibuprofen (64-70%) than that in the activated sludge process (57-60%). These could be attributed to adaptation of the biofilms to the pharmaceutical residues. As for antibiotics, 2 mg L^{-1} of lincomycin was found posing a significant inhibition on nitrification activity of activated sludge process (Carucci et al. 2006). Meanwhile, recent study showed that lincomycin could not inhibit the bacterial activity in the *Staphylococcus aureus* biofilms at concentrations up to 220 mg L^{-1} (Tote et al. 2009). Ma et al. (2009) also reported a good performance of two biofilm reactors for treating the high-strength terramycin production wastewater in term of COD removal, but the removal of terramycin was not determined. It appears that biofilm technology is a potential alternative for removing antibiotic residues from wastewaters. It should be noted that information about the biofilms applied for the antibiotic-bearing wastewater treatment is still very limited, and further study is highly desirable.

2.3.3 Advanced Oxidation

Advanced oxidation processes (AOPs) typically involve the generation and use of the hydroxyl free radical ($HO^\bullet$) as a strong oxidant to destroy complex non-biodegradable organic constituents in wastewater. Except for the conventional oxidants, such as oxygen, ozone and chlorine, other reagents able to yield $HO^\bullet$ include UV-H_2O_2, Fenton's reagent and ultrasound. These processes involve the generation of free radicals via chemical (e.g. by O_3, O_3/H_2O_2, Fe^{2+}/H_2O_2), photochemical (e.g. by UV /H_2O_2, UV /O_3), electrochemical (e.g. by electrons) and photocatalytic (e.g. by UV /TiO_2) reactions. It has been reported that combined AOPs are more effective than any of the individual agents (e.g., ozone, UV, hydrogen peroxide) (Metcalf & Eddy 2004).

So far, AOPs have not been widely used in WWTPs, due to the high cost of installation and operation. Meanwhile, recent studies show that oxidation could be successfully used as a pre-treatment step to improve the biodegradability of antibiotic wastewater. Balcioglu and Otker (2003) investigated the ozonation and O_3/H_2O_2 for treating three synthetic pharmaceutical formulation wastewaters containing a veterinary antibiotic and two human antibiotics. It was found that biological/chemical oxygen demand ratio was increased from 0.077 to 0.38 for the veterinary antibiotic-bearing wastewater, and 0 to 0.1 and 0.27 for human antibiotic I- and human antibiotic II-bearing wastewater, respectively. In addition, more advanced oxidation methods (e.g. O_3/OH^-, H_2O_2/UV, Fe^{2+}/H_2O_2, Fe^{3+}/H_2O_2, Fe^{2+}/H_2O_2/UV and Fe^{3+}/H_2O_2/UV) were also employed for the pre-treatment of the real penicillin formulation effluent (Arslan-Alaton and Dogruel 2004). Among these oxidation methods, H_2O_2/UV-C treatment was relatively less effective due to the fact that H_2O_2 photo-dissociation is seriously inhibited in the presence of other strong UV absorbers, such as the penicillin formulation effluent.

Another major concern about oxidation is the high potential toxicity of the by-products. Adams et al. (2002) reported that the oxidations by ozone and chlorine were both effective to remove the antibiotics studied. However, the chlorination and ozonation by-products and pathways, as well as the pharmacological properties of these by-products, were suggested to be further examined in their study. De Witte et al. (2009) identified the major oxidation by-product of ciprofloxacin which was desethylene ciprofloxacin and still an antimicrobial-active substance.

2.3.4 Membrane Technology

Membrane technology is the separation method using the selective membrane that may allow the passage of certain constituents and retain other constituents present in liquid. Membrane processes are classified as microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), reverse osmosis (RO), dialysis, and electrodialysis (ED). The general characteristics and the typical operating ranges of each membrane process are summarized in Table 2.7.

Table 2.7 General characteristics of membrane processes (Metcalf & Eddy 2004).

Membrane process	Driving force	Separation mechanism	Structure (pore size)	Operating range, µm	Permeate description
MF	Hydrostatic pressure or vacuum in open vessels	Sieve	Macropores (>50nm)	0.08-2.0	Water + dissolved solutes
UF	Hydrostatic pressure difference	Sieve	Mesopores (2-50nm)	0.005-0.2	Water + small molecules
NF	Hydrostatic pressure difference	Sieve+solution/ diffusion+exclusion	Micropores (<2nm)	0.001-0.01	Water + very small molecules, ionic solutes
RO	Hydrostatic pressure difference	Solution /diffusion+exclusion	Dense (<2nm)	0.0001-0.001	Water, very small molecules, ionic solutes
Dialysis	Concentration difference	Diffusion	Mesopores (2-50nm)	—	Water + small molecules
ED	Electromotive force	Ion exchange with selective membrane	Micropores (<2nm)	—	Water + ionic solutes

In view of the molecular size of antibiotics, UF, NF and RO would be applicable for the removal of antibiotics from water. As early as in 1978, a reverse osmosis (RO) system was developed and successfully applied for hyperfiltration of the industrial wastewater from a pharmaceutical plant producing antibiotics (Wagner 1978). Zhu et al. (2003) reported separation and recovery of clindamycin from clindamycin fermentation wastewater with nanofiltration (NF) membranes. Results showed that the clindamycin rejection and recovery efficiency was higher than 94% and 86.5%, respectively.

Combined membrane system with several membrane units are also reported for the treatment of antibiotic wastewater. In China, a new pre-treatment approach using RO+UF was proposed for the treatment of an oxytetracycline (OTC) waste liquor (Li et al. 2004). In Croatia, a RO+NF system was used to remove a veterinary antibiotics from the wastewater of a pharmaceutical manufacturing plant (Dolar et al. 2009). It was shown that the rejection of the examined antibiotics by the selected RO and the tight NF membranes was acceptably high, exceeding 98.5% in most cases. However, it should be noted that a concentrated reject stream with antibiotic residues would still require additional treatment.

2.3.5 Coagulation/Flocculation/Sedimentation

Coagulation/flocculation/sedimentation, by which chemicals are added to water to cause destabilization of colloidal particles, allowing aggregation through flocculation, followed by sedimentation process, is a mean to remove soluble species. In natural system, the antibiotics would be in contact with natural colloidal matters (e.g., clays) for extended periods, providing the opportunity for potential adsorption of antibiotics on colloidal matter to occur. If adsorbed on colloids, the antibiotics could be co-removed in a coagulation/flocculation/sedimentation process. However, in the work of Adams et al. (2002), no significant removal of the antibiotics studied was observed with alum or ferric salt as coagulant. So far, this technology for the antibiotic removal has not yet been proven.

2.3.6 Combined Technologies

Zhang et al. (2006) integrated powdered activated carbons (PACs), ultrafiltration (UF) and reverse osmosis (RO) for treating the tetracycline (TC) production wastewater, in which UF was used as a pre-treatment and reduced the fouling in RO. Results showed that the UF-RO performance was improved by adding PACs to the wastewater. Baumgarten et al. (2007) used membrane bioreactor reactors (MBRs) to pre-treat the wastewater containing antibiotics e.g. floxacins and their synthetic precursor compounds. PAC was also added into one MBR and it was observed that addition of PAC greatly increased the elimination rate as compared to the MBR free of PAC. The study by Watkinson et al. (2007) showed the removal of 28 human and veterinary antibiotics in an integrated system with activated sludge, MF and RO in sequence. About 87%, 43%, and 94% of the antibiotics removal was achieved in the activated sludge, MF and RO units, respectively. All these findings show that the high removal efficiency of antibiotics can be achieved through the combination of the conventional biological process and other water treatment technologies, which in turn enlightens the way for the further study of antibiotic-bearing wastewater treatment.

2.3.7 Biofilm-covered GAC (BGAC)

Biofilm-covered granular activated carbon (BGAC) has the combine merits of both adsorption and biodegradation. Specifically, the rough surface of GAC provides excellent shelter from fluid shear forces for colonization of microorganisms (Characklis 1973; Weber et al. 1978), and GAC adsorption may attenuate high or toxic incident influent concentrations of pollutant (Khodadoust et al. 1997). Meanwhile, attached microorganisms onto GAC could persistantly degrade the organic compounds, resulting in an in situ bio-regeneration of GAC (Aktas and Cecen 2007). Due to these advantages, GAC and PAC have been widely used as carrier materials for biofilm development. Fan et al. (1987) used PAC to treat wastewater containing phenol at concentration of 100 mg L^{-1} in an airlift bioreactor, and a high removal efficiency (>99%) of phenol was achieved. BGAC was also employed to remove atrazine from wastewater at the concentration of 5 to 25 mg L^{-1} in the fluidized reactor (Herzberg et al. 2004). The results showed that the atrazine biodegradation rate by BGAC was higher than that using the non-adsorbing carrier. This means that the release of organics from GAC to biofilm would strengthen the biodegradation capability of BGAC. Similarly, other studies showed that BGAC can remove a wide variety of recalcitrant pollutants from wastewater with high efficiency (Khodadoust et al. 1997;Carvalho et al. 2001; Ong et al. 2008a; Mello et al. 2010). Although there are many studies about BGAC for the removal of organic pollutants, the application of BGAC for treating antibiotic wastewater is not reported within the scope of the literature.

2.4 SUMMARY

This review shows that antibiotics have become an emerging pollutant that has to be removed during the wastewaters treatment. Various technologies have been employed for such purpose, e.g. adsorption, biological process, advanced oxidation, and membrane separation. It can be seen that a single technology is inefficient in handling the large amounts of antibiotic-bearing wastewater. Nowadays, both adsorption and biological process are the two well-established technologies for the treatment of recalcitrant wastewater, thus appear potential for antibiotic wastewater.

The main drawback of adsorption is that antibiotics are only physically transferred from one phase to another without the breakup of their functional structures; whereas biodegradation may be implicated by the antimicrobial property of antibiotics. It appears from this review that the knowledge about the treatment of antibiotic-bearing wastewater is rather scarce and definitely in-depth and systematic study is urgently needed. In such situation, this study aimed to develop a novel combined adsorption-biodegradation system with the capability of self-regeneration for high-efficiency treatment of antibiotic-bearing wastewater.

CHAPTER 3

ADSORPTION OF ANTIBIOTICS BY GAC: ISOTHERM, THERMODYNAMICS AND KINETICS

3.1 INTRODUCTION

As reviewed in Chapter 2, adsorption has a great potential for the removal of soluble pollutants including antibiotics. The performance of a carbon adsorption process is determined by the adsorption capacity and kinetics of a target chemical. So far, adsorption isotherms have been well documented, while there is still a strong debate on adsorption kinetics. For example, in almost all adsorption studies, two empirical kinetic models, i.e., first- and second-order rate equations, have been used to fit the experimental data, and one is often claimed to be more appropriate than the other according to the marginal difference in correlation coefficients. As noted by Rudzinski and Plazinski (2006), in the past decades few work has been done to clearly address the theoretical origins of these two commonly used kinetic equations for adsorption. Therefore, this study attempted to investigate the isotherms, kinetics and thermodynamics of antibiotics adsorption by activated carbon, while special focus would be placed to explore the theoretical origins of first- and second-order rate equations for adsorption. For this purpose, three widely used β-lactam antibiotics (PCG, AMP and CPC) were chosen as the model antibiotics, whereas granular activated carbon (GAC) was selected as the adsorbent as well as a biocarrier for future biofilm development (Chapter 4).

3.2 MATERIALS AND METHODS

3.2.1 Adsorbent and Antibiotics

Granular activated carbon (GAC) from Calgon Carbon Corporation, USA, was used as the adsorbent with a mean size of 2.8 mm, an apparent density of 450 kg m^{-3} and a particle density of 650 kg m^{-3}. The GAC was carefully rinsed with distilled water and dried at 103°C overnight before use.

In this study, three representative β-lactam antibiotics, namely penicillin G (PCG), ampicillin (AMP) and cephalosporin C (CPC) from Sigma-Aldrich Pte Ltd, Singapore, were used as the model antibiotics as illustrated in Figure 3.1. Values of log K_{ow} are 1.83, 1.35 and -1.46 for PCG, AMP and CPC respectively. Moreover, respective water solubilities are 25, 50 and 0.209 g L^{-1} for PCG, AMP and CPC.

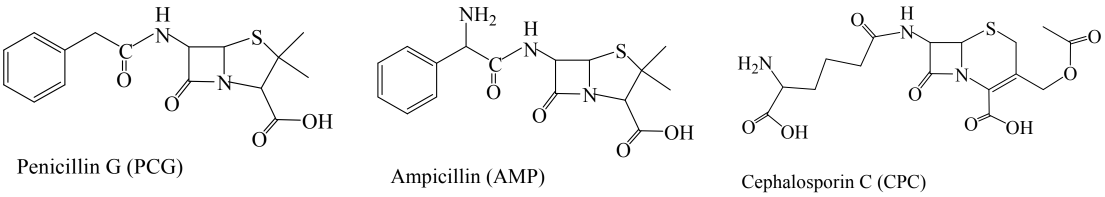

Penicillin G (PCG)
Ampicillin (AMP)
Cephalosporin C (CPC)

Figure 3.1 Molecular structures of PCG, AMP and CPC.

3.2.2 Adsorption Experiments

Equilibrium experiments were performed with 1.0 to 10.0 grams of GAC in 250 mL of the antibiotic solutions prepared with distilled water at various concentrations in a temperature controlled shaking thermostat at the desired temperature of 25°C, 30°C, 35°C and 40°C, respectively. The adsorption time was 72 h. The samples were filtered through a 0.2 μm membrane filter, and the filtrates were then analyzed for antibiotic concentration until the adsorption equilibrium. The amount of adsorbed antibiotic per gram of GAC (q_e) at equilibrium was calculated as follows:

$$q_e = \frac{C_0 - C_e}{X} \tag{3.1}$$

in which, C_0 and C_e are the initial and equilibrium antibiotic concentrations (mg L^{-1}) respectively, and X is the GAC dosage (g L^{-1}).

Kinetic experiments were carried out in a 2-liter Erlenmeyer flask on a shaker at 25ºC. GAC at the dosage of 1.0 to 10.0 grams was added into 1 L of antibiotic solution with a preset initial concentration of 10 to 1500 mg L^{-1} for PCG; 10 to 1000 mg L^{-1} for AMP; and 10 to 100 mg L^{-1} for CPC, respectively. 2 mL of the solution was collected for the concentration analysis at different time intervals.

3.2.3 Analytical Methods

The specific surface area and the pore diameter of the GAC were determined by the nitrogen-based Brunauer-Emmett-Teller (BET) method with the Micromeritics software. Fourier transform infrared (FTIR) spectra of samples were obtained from the BioRad Excalibur Series FTS 3000 spectrometer with deuterated triglycine sulfate (DTGS) detector, using a pellet technique. The pellets were prepared with KBr powder. In this study, the FTIR spectrometer was set at resolution of 4 cm^{-1}, spectral range of 4000 cm^{-1} to 500 cm^{-1}, and sensitivity of 1. Spectra were obtained by averaging 30 scans.

Concentrations of antibiotics were determined by high-performance liquid chromatography (HPLC, Perkin Elmer Series 200, USA) with a UV detector at 220 nm. For this purpose, an anion exchange column with BioBasic AX 150 mm×4.6 mm and 5 μm particle size (Thermo, Massachusetts, USA), was used to separate antibiotics from the solution. 10 mM of the mixed ammonia acetate (CH_3COONH_4) and acetonitrile (CH_3CN, ACN) solution in a ratio of 90:10 by volume, was used as the mobile phase with the flow rate of 1 mL min^{-1}. The column temperature was set to 35ºC and 20 μL for injection volume. In case where concentration was below the detection limit, 10 mL of sample was pre-concentrated using the 30 mg Waters Oasis HLB cartridge (Waters, Massachusetts, USA). Before use, the cartridge was pre-conditioned with 5 mL of ACN and water in succession. A wash step with 1 mL of 5% ACN (v/v) was applied after the sample loading. The cartridge was air dried for about 5 minutes under vacuum to remove excess water. The analytes retained were eluted with 1 mL ACN, and the final elution volume was approximately 1 mL. Antibiotics in the samples can be pre-concentrated by 10 times according to this procedure.

3.3 RESULTS

3.3.1 Characterization of GAC

The physicochemical properties of GAC before and after antibiotics adsorption at 25°C are shown in Table 3.1. The comparison with virgin GAC showed the reduction of total pore volume, external surface area and BET surface area in the antibiotic-loaded carbon, which can be attributed to accumulation of antibiotics in the GAC. In fact, the values of micropore volume and surface reflect possible adsorption sites of solutes. For PCG, adsorption mostly took place in micropores, which also accounted for a slight increase in the average pore diameter from 2.865 nm of virgin carbon to 2.905 nm of PCG-loaded carbon. As for AMP and CPC, they were mainly adsorbed in the pores with larger diameter, as the average pore diameter of the used carbon was smaller than that of the virgin carbon.

Table 3.1 Characteristics of GAC at 25°C.

GAC sample	Average pore diameter (nm)	Total pore volume (cm^3 g^{-1})	Micropore volume (cm^3 g^{-1})	Surface area (m^2 g^{-1})		
				External	Micropore	BET surface area
Virgin GAC	2.865	0.7783	0.09390	899.3	187.2	1087
Saturated with PCG	2.905	0.551	0.03995	675.9	83.69	759.6
Saturated with AMP	2.635	0.4890	0.1062	536.5	205.9	742.3
Saturated with CPC	2.568	0.6702	0.1020	843.7	200.1	1044

The FTIR spectra are generally used to identify the functional groups that are capable of adsorbing organic compounds. Figure 3.2 shows the FTIR spectra of GAC before and after adsorption of antibiotics at 25°C. The common structure of the PCG, AMP and CPC is the four-membered (β) lactam ring fused to another thiazole ring. Therefore, in FTIR spectra of the used GAC, the peak at around 1700 cm^{-1} represents the C=O stretching vibration of the β-lactam ring; the peak at around 3480 cm^{-1} represents the N-H stretching vibration of the β-lactam ring. Furthermore, the peak at around 650 cm^{-1} in the spectra of PCG-loaded GAC and AMP-loaded GAC suggest the C-H bending vibration of the benzene ring. Meanwhile, this peak did not appear in the spectra of CPC-loaded GAC due to the absence of the benzene ring in the CPC molecular structure. The intermediate portion of the spectrum of 1300-900 cm^{-1}, is usually referred to as the "fingerprint" region. The absorption pattern in this region is rather complex because the stretching vibration of all single bonds and vibration of molecular skeleton would take place. Compared to the spectrum of virgin GAC, absorbance in the spectra of the antibiotic-loaded GAC became more intense, showing that antibiotics were adsorbed on the GAC surface through chemical bonds and subsequent formation of the more functional groups.

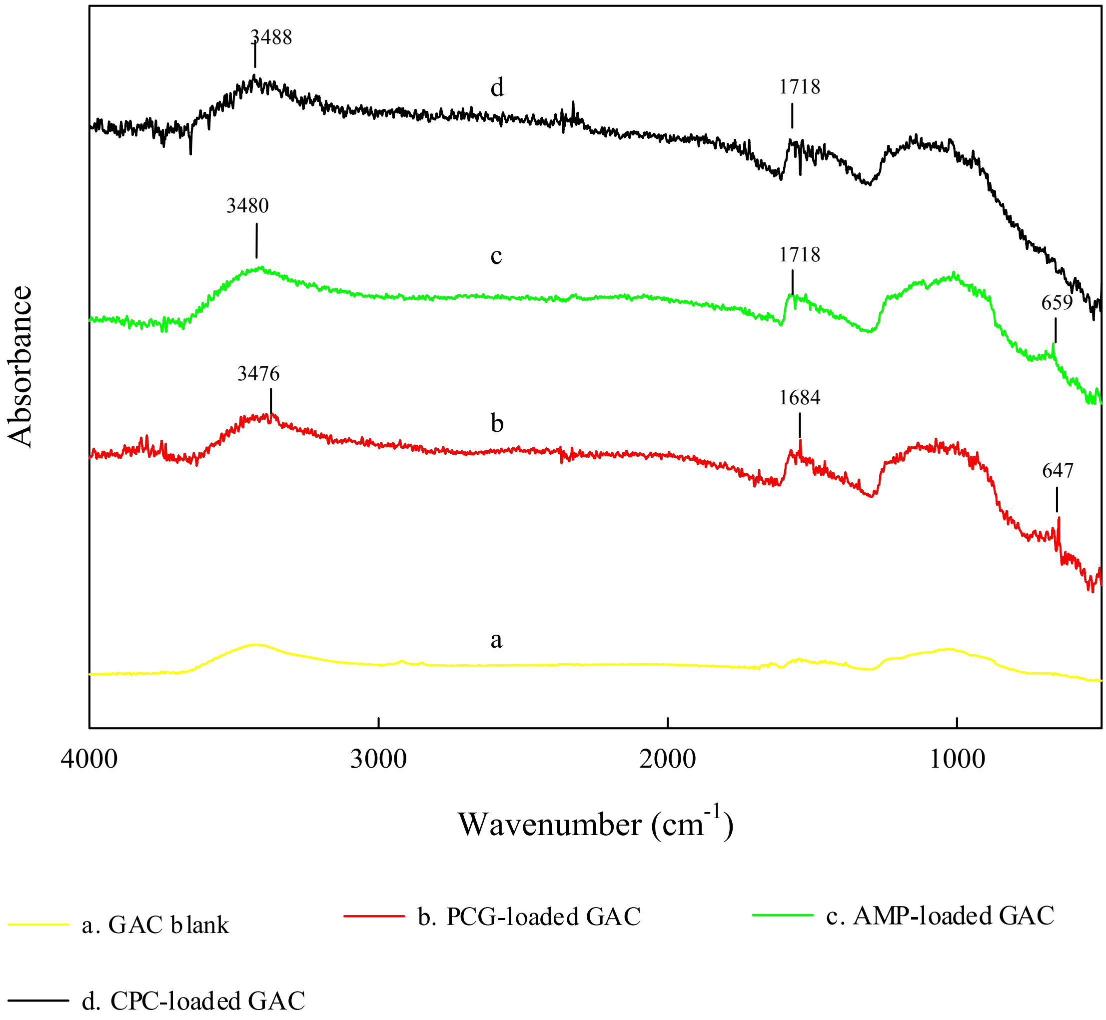


Figure 3.2 FTIR spectra of virgin and antibiotic-loaded GAC at 25°C.

3.3.2 Adsorption Isotherm

Equilibrium analysis is the fundamental study to evaluate the adsorption capacity of the adsorbent. A variety of different isotherm equations have been developed, some of which have a theoretical basis and some are of a more empirical nature. In this study, the most commonly used Langmuir and Freundlich isotherms were employed to describe the equilibrium data obtained in the adsorption experiments of three antibiotics by GAC at four different temperatures, respectively (Figures 3.3 to 3.5).

Langmuir Isotherm

The Langmuir model was originally developed to represent chemisorption on a set of distinct localized adsorption sites with the following expression:

$$q_e = q_m \frac{K_L C_e}{K_L C_e + 1} \tag{3.2}$$

in which, K_L is the Langmuir adsorption constant; C_e and q_e are solute concentration and adsorption capacity at equilibrium, respectively; and q_m is the maximum adsorption capacity. Eq. 3.2 can be rearranged to

$$\frac{\theta_e}{1-\theta_e} = K_L C_e \tag{3.3}$$

in which θ_e is the coverage degree of adsorbent surface at equilibrium ($\theta_e = q_e/q_m$, $0 \le \theta_e \le 1$).

Freundlich Isotherm

The Freundlich isotherm is proposed by assuming a heterogeneous surface with a non-uniform distribution of heat of adsorption over the surface, and is often expressed as follows:

$$q_e = K_F C_e^{1/n} \tag{3.4}$$

in which, K_F is the Freundlich constant indicating the adsorption capacity, and 1/n is the heterogeneity factor indicating adsorption intensity.

Experimental data at equilibrium were fitted to the Langmuir and Freundlich isotherm equations, as shown in Figures 3.3 to 3.5, respectively. The constants involved were estimated using a non-linear regression method provided by Matlab 7, and are summarized in Table 3.2.

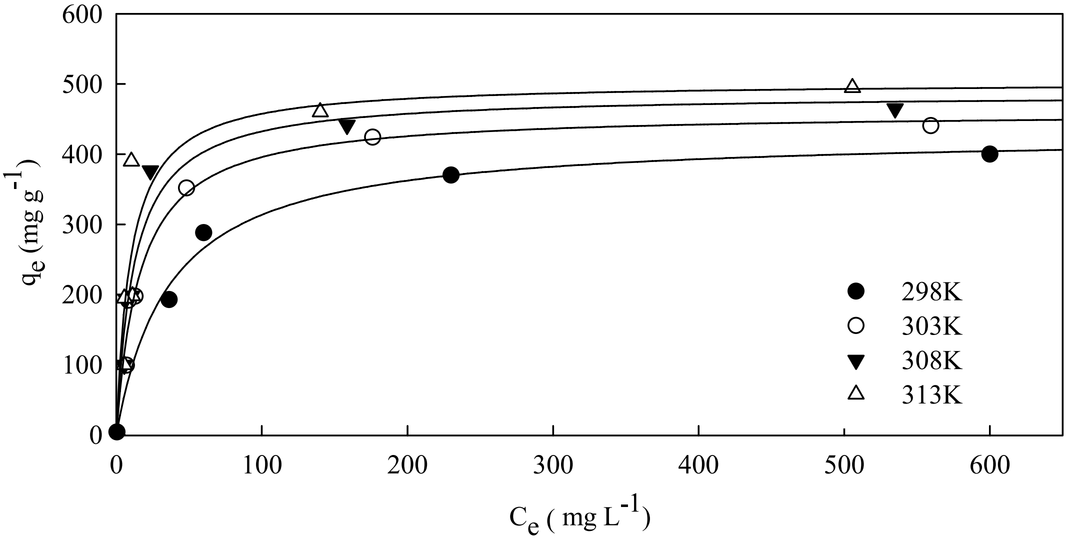


Figure 3.3 Equilibrium data of PCG adsorption by GAC at different temperatures, solid line: prediction by Langmuir isotherm.

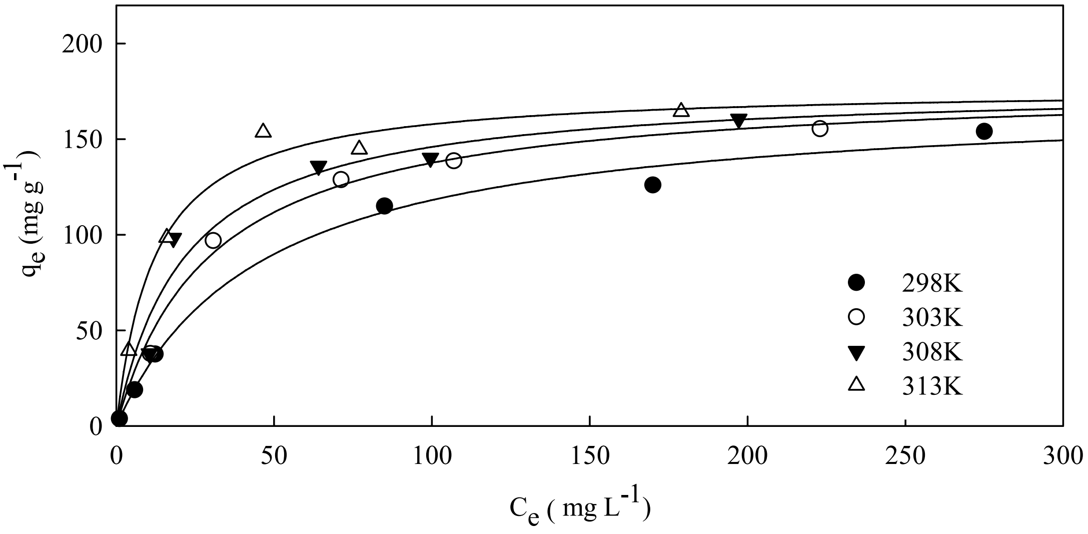


Figure 3.4 Equilibrium data of AMP adsorption by GAC at different temperatures, solid line: prediction by Langmuir isotherm.

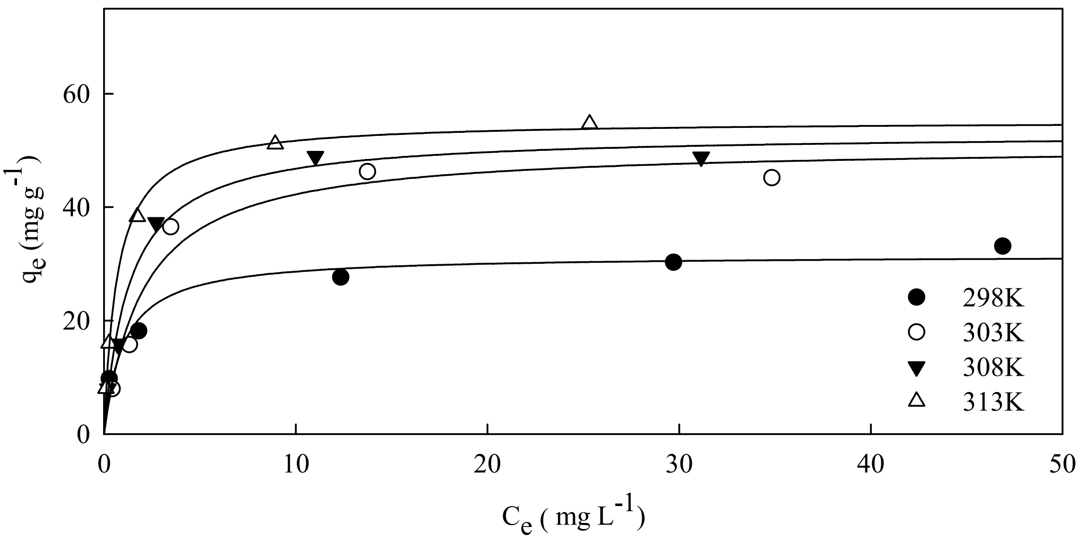


Figure 3.5 Equilibrium data of CPC adsorption by GAC at different temperatures, solid line: prediction by Langmuir isotherm.

Table 3.2 Equilibrium constants for the adsorption of antibiotics by GAC.

Antibiotics	T (K)	Freundlich isotherm			Langmuir isotherm		
		K_F	n	R^2	q_m (mg g^{-1})	K_L (L mg^{-1})	R^2
PCG	298	207.3	3.80	0.9144	427.3	0.028	0.9931
PCG	303	261.5	4.44	0.8334	460.2	0.061	0.9711
PCG	308	288.2	4.77	0.7681	485.6	0.081	0.9355
PCG	313	309.0	4.93	0.6920	502.7	0.102	0.9856
AMP	298	65.49	2.24	0.9680	164.2	0.023	0.9981
AMP	303	92.32	3.01	0.8777	177.1	0.033	0.9831
AMP	308	100.7	3.16	0.8320	178.0	0.045	0.9205
AMP	313	117.4	3.72	0.8410	179.0	0.082	0.9875
CPC	298	33.07	3.28	0.7851	33.67	0.46	0.8543
CPC	303	53.62	3.64	0.7996	50.9	0.49	0.9584
CPC	308	59.14	3.86	0.8460	63.2	0.75	0.9929
CPC	313	67.46	4.03	0.9032	71.9	1.46	0.9879

Table 3.2 shows that the Langmuir isotherm can provide a better description for adsorption data of PCG, AMP and CPC than the Freundlich isotherm at all the temperatures studied. The values of q_m from the Langmuir isotherm indicate that GAC has a substantial adsorption capacity for three β-lactam antibiotics studied, and the adsorption capacity of antibiotics by GAC is in the order of PCG>AMP>CPC.

3.3.3 Adsorption Thermodynamics

Entropy and energy factors should be considered in order to determine whether the adsorption of antibiotics by GAC would occur spontaneously. The Gibbs free energy indicates the degree of spontaneity of the adsorption process and higher negative value reflects a more energetically favorable adsorption. The Gibbs free energy change (ΔG°) of adsorption is defined as

$$\Delta G^{o} = -RT \ln K_L \tag{3.5}$$

It is also related to the change in entropy, ΔS° and the heat of adsorption, ΔH° at a given temperature in a way such that:

$$\Delta G^{o} = \Delta H^{o} - T\Delta S^{o} \tag{3.6}$$

Combining Eqs. 3.5 and 3.6 yields,

$$\ln K_L = -\frac{\Delta H^{o}}{RT} + \frac{\Delta S^{o}}{R} \tag{3.7}$$

in which ΔG° is the free energy change (kJ mol^{-1}); ΔH° is the change in enthalpy (kJ mol^{-1}); ΔS° is the entropy change (kJ mol^{-1} K^{-1}); T is the absolute temperature (K); and R is the universal gas constant (8.314 $Jmol^{-1}$ K^{-1}). Thus, ΔH° and ΔS° can be determined by the slope and the intercept of the linear Van't Hoff plot, i.e. ln K_L versus 1/T.

The equilibrium constants obtained from the Langmuir isotherm at 298, 303, 308 and 313K were used to calculate ΔG°, ΔH° and ΔS° for adsorption of PCG, AMP and CPC by GAC, respectively (Table 3.3).

Table 3.3 Thermodynamic parameters for the adsorption of antibiotics by GAC.

Adsorbent	ΔH° (kJ mol^{-1})	ΔS° (J mol^{-1} K^{-1})	ΔG° (kJ mol^{-1})			
			298K	303K	308K	313K
PCG	64.8	295.3	-22.8	-25.2	-26.3	-27.3
AMP	64.4	290.8	-22.4	-23.7	-24.9	-26.9
CPC	60.3	301.9	-30.1	-30.8	-32.4	-34.7

Table 3.3 shows that ΔH° and ΔS° have positive values, and ΔG° has negative values. The positive ΔH° values imply that the adsorption of three antibiotics on GAC is endothermic. In addition, the value of q_m in Table 3.2 increased with the increase in temperature, which confirms that the endothermic nature of the adsorption of three antibiotics by GAC. The values of ΔH° were 64.8, 64.4 and 60.3 kJ mol^{-1} for PCG, AMP and CPC adsorption on GAC, respectively. Basically, the heat evolved during the physical adsorption is of the same order of magnitude as the heat of condensation, i.e., 10 to 20 kJ mol^{-1}, meanwhile the heat of chemisorption generally falls into the range of 40 to 400 kJ mol^{-1} (Bansal and Goyal 2005). Therefore, it appears that chemisorption would be the predominant mechanism of the antibiotics adsorption by GAC. The positive values of ΔS° suggest the increased randomness at the solid-solution interface with some structural changes in the adsorbate/adsorbent and antibiotic affinity to GAC. In fact, the positive ΔS° value is often referred to an increase in the degree of freedom of the adsorbed species. The negative values of ΔG° indicate the degree of spontaneity of the adsorption process, i.e. the more negative value of ΔG°, the more energetically favorable adsorption was. It seems that the three antibiotics adsorption by GAC would be spontaneous in the nature and the spontaneity would be increased with temperature (Table 3.3).

3.3.4 Adsorption Kinetics

Langmuir kinetics

The chronologically first theoretical kinetic model proposed for adsorption is the one developed by Langmuir in 1918 for description of the kinetics of gases adsorption onto solid surfaces and adopted later for the solid/solution systems. According to Langmuir (1918), adsorption can be regarded as a reversible reaction between adsorbent and solute:

$$A + B \underset{k_d}{\overset{k_a}{\longleftrightarrow}} AB \qquad (3.8)$$

in which A is the solute; B is the adsorption site on the adsorbent surface; AB is the complex formed; k_a is adsorption rate constant and k_d is desorption rate constant. The proportion of the surface occupied by solute (θ) is defined as

$$\theta = \frac{q}{q_m} = \frac{C_0 - C}{q_m X} \qquad (3.9)$$

in which q is the adsorption capacity of the adsorbent at time t, and q_m is its maximum value. C_0 and C are the respective concentrations of adsorbate in solution at time zero and time t, while X represents the dosage of adsorbent. Eq. 3.9 can be rearranged to

$$C=C_0-q_mX\theta \qquad (3.10)$$

The adsorption and desorption rates in Eq. 3.8 can be written as

$$r_a=k_aC(1-\theta) \qquad (3.11)$$

$$r_d=k_d\theta \qquad (3.12)$$

It should be pointed out that Eqs. 3.11 and 3.12 are developed based on the assumption that the forward adsorption rate is first-order with respect to C and (1-θ), and the desorption follows the first-order regarding the sites occupied (θ) as well. Thus, the overall rate equation can be expressed as follows:

$$\frac{d\theta}{dt} = r_a - r_d = k_a C(1-\theta) - k_d\theta \tag{3.13}$$

Eq. 3.13 has been often referred to as the Langmuir kinetics (Langmuir 1918).

First-order rate equation

The first-order equation or the so-called Lagergren (1898) equation is given by Eq. 3.14.

$$\frac{dq}{dt} = k_1^{'}(q_e - q) \tag{3.14}$$

in which q is the adsorption capacity at time t; q_e is its equilibrium value; and k_1’ is a constant. The first-order rate equation indeed is in line with the concept of linear driving force.

Second-order rate equation

Analogue to the first-order rate equation, the second-order rate equation is often expressed in the form of Eq. 3.15, which was first proposed by Blanchard et al. (1984). Thereafter, it has been most frequently employed to analyze adsorption data obtained from various experiments using different adsorbates and adsorbents.

$$\frac{dq}{dt} = k_2^{'}(q_e - q)^2 \tag{3.15}$$

in which k_2’ is a constant.

The adsorption experiments of three model antibiotics were conducted at a fixed GAC dosage of 5 g L^{-1} at 25°C. Adsorption data of PCG, AMP and CPC obtained at different initial concentrations were fitted to the Langmuir kinetics, first- and second-order rate equations, respectively. It appears from Figures 3.6 to 3.8 that the model predictions are in good agreement with the experimental data, and the prediction by the Langmuir kinetics always falls in between the predictions by the first- and second-order rate equations.

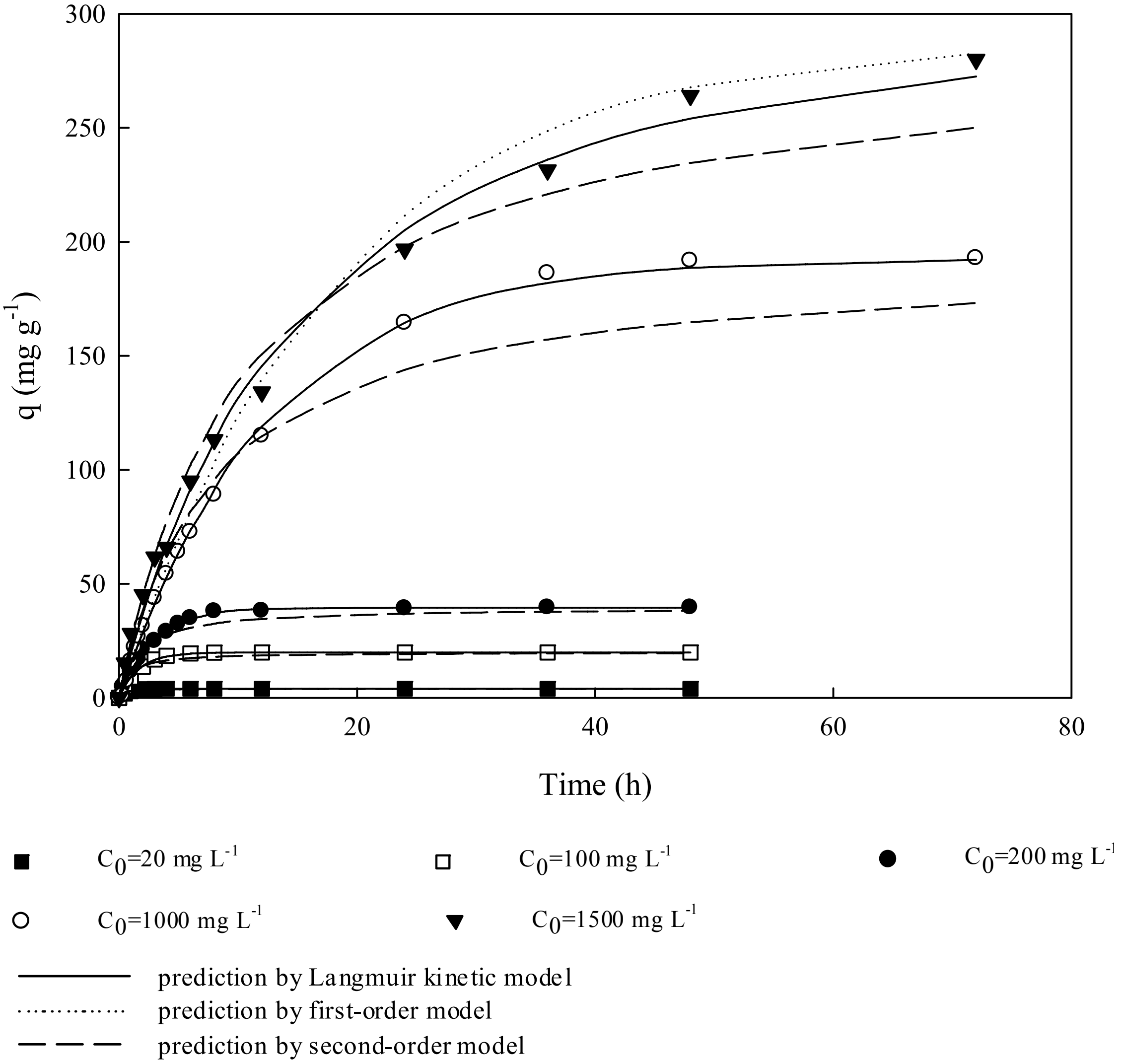


Figure 3.6 Adsorption kinetics of PCG by GAC at various initial PCG concentrations.

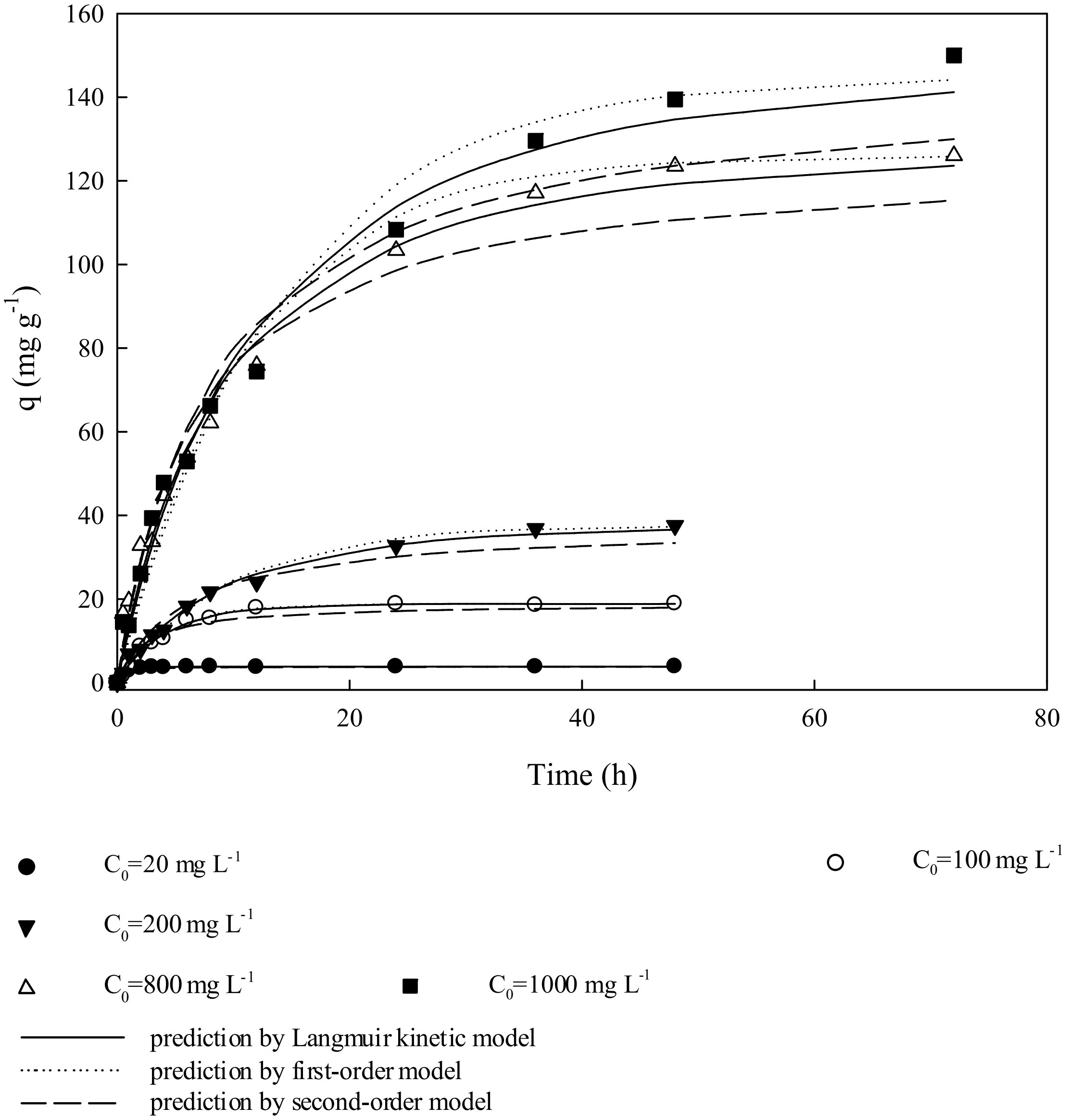


Figure 3.7 Adsorption kinetics of AMP by GAC at various initial AMP concentrations.

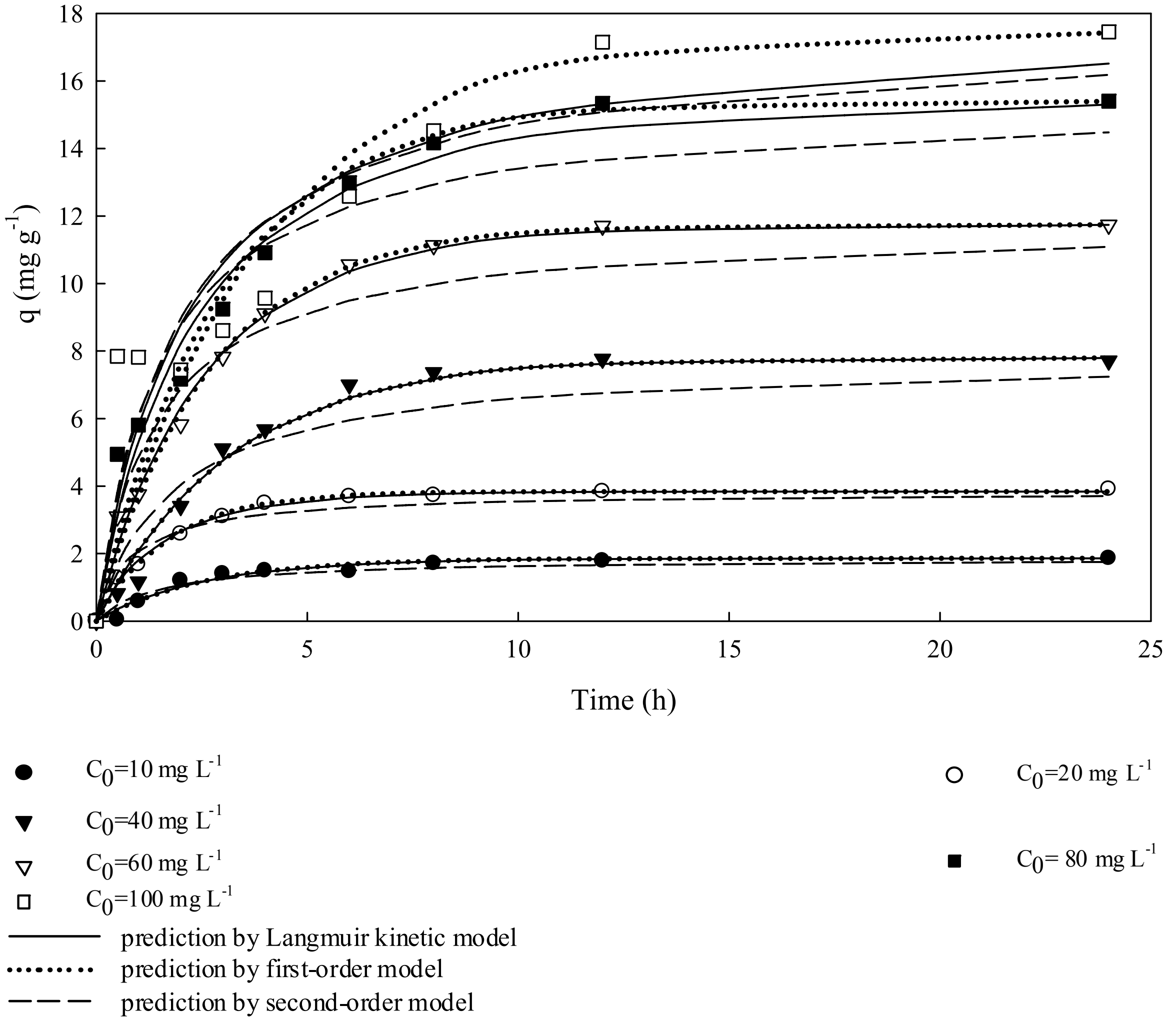


Figure 3.8 Adsorption kinetics of CPC by GAC at various initial CPC concentrations.

In previous studies of adsorption kinetics, the correlation coefficient (R) or R^2 has been often used to compare how good one kinetic equation is over the others. However, it appears from Tables 3.4 that R^2 may not be sensitive enough to distinguish the goodness of curve fittings by first- and second-order as well as the Langmuir kinetic equations simply due to the fact that the observed difference in R is too small to make any firm conclusion. Therefore, R itself can not provide sufficient and reliable information about how good one kinetic equation is against the others.

Table 3.4 Comparison of prediction errors from different adsorption kinetic equations.

No.	Antibiotics	C_0	1^{st}-order		2^{nd}-order		Langmuir kinetics	
		(mg L^{-1})	R^2	f	R^2	f	R^2	f
1	PCG	20	0.9999	0.999	0.9627	0.0034	0.9999	1.00
2	PCG	100	0.9995	0.999	0.9522	0.010	0.9995	1.00
3	PCG	200	0.9981	0.999	0.9584	0.046	0.9981	1.00
4	PCG	1000	0.9986	0.999	0.9617	0.998	0.9986	1.00
5	PCG	1500	0.9848	0.389	0.9781	0.692	0.9941	1.00
6	AMP	20	0.9964	0.924	0.9720	0.120	0.9966	1.00
7	AMP	100	0.9910	0.942	0.9629	0.228	0.9916	1.00
8	AMP	200	0.9870	0.606	0.8328	0.248	0.992	1.00
9	AMP	800	0.9758	0.515	0.7725	0.438	0.9876	1.00
10	AMP	1000	0.9780	0.606	0.8234	0.392	0.9866	1.00
11	CPC	10	0.9567	0.995	0.9267	0.582	0.9571	1.00
12	CPC	20	0.9929	0.663	0.9630	0.127	0.9953	1.00
13	CPC	40	0.9834	0.986	0.9139	0.190	0.9837	1.00
14	CPC	60	0.9912	0.913	0.9551	0.179	0.9920	1.00
15	CPC	80	0.9600	0.673	0.9553	0.601	0.9731	1.00
16	CPC	100	0.7880	0.732	0.8437	0.993	0.8448	1.00

Liu and Wang (2008) proposed a statistic evaluation parameter, namely relative goodness (f) of curve fitting which is defined according to the prediction error square (σ^2):

$$f = \frac{(\sigma^2)_{min}}{\sigma^2} \tag{3.16}$$

The term σ^2 is given by

$$\sigma^2 = \frac{\sum (Y_k - \hat{Y}_k)^2}{n - p} \tag{3.17}$$

in which Y_k and $\hat{Y}_k$ are experimental data and regressed values, n and p are numbers of experimental data and regressed coefficients, respectively (Montgomery et al. 2003). The $(\sigma^2)_{min}$ in Eq. 3.16 is the minimum value among all σ^2 determined by different kinetic equations (e.g. Eqs. 3.13 to 3.15).

It can be seen from Table 3.4 that all the R^2 values were aggregated into a very narrow range, but the values of the relative goodness (f) of curve fitting largely varied from 0.0034 to 1.00. This means that f can provide an excellent statistical comparison among all the kinetic equations studied (Eqs. 3.13 to 3.15), but R^2 is unable to offer any insightful information in this regard. Compared to the preset-order kinetic equations (Eqs. 3.13 to 3.15), it appears from f values that the Langmuir kinetic equation (Eq. 3.13) offers the best description of the adsorption data. In almost all previous studies of adsorption kinetics, the preset-order (e.g. first- or second- order) equation (Eqs. 3.14 or 3.15) has been directly chosen to fit adsorption data without any explanation about the rational behind. In addition, goodness of Eqs. 3.14 and 3.15 in fitting adsorption data is often compared according to the magnitude of correlation coefficient (Liu et al. 2003; Erdem and Ozverdi 2006; Kiran et al. 2006). Nevertheless, it appears from Table 3.4 that a high R^2 value does not necessarily mean a high f value. For example, for PCG adsorption at C_0=1500 mg L^{-1}, the R^2 values determined from the curve fittings by Eqs. 3.14 and 3.15 were as high as 0.9848 and 0.9781 respectively, while the corresponding f values were as low as 0.389 and 0.692, respectively. These in turn suggest that both Eqs. 3.14 and 3.15 would be equally poor over the Langmuir kinetic equation in describing these adsorption data. In such a case, the R^2-based comparison between the first- and second-order rate equations would be less meaningful.

3.3.5 Theoretical Origins of First- and Second-order Rate Equations for Adsorption

In nearly all studies about adsorption kinetics, the first- and second-order rate equations have been frequently employed to describe the adsorption kinetic data. However, limited effort has been made to validate these two empirical rate equations for adsorption, i.e., theoretical basis of the first- and second-order rate equations for adsorption is still unknown.

3.3.5.1 Transformation of Langmuir kinetics

The overall rate equation for adsorption can be expressed as Eq. 3.13 which has often been used for derivation of the Langmuir isotherm equation at $d\theta/dt=0$.

Inserting Eqs. 3.10 to 3.12 into Eq. 3.13 yields

$$\frac{d\theta}{dt} = k_a q_m X\theta^2 - (k_a C_0 + k_a q_m X + k_d)\theta + k_a C_0 \tag{3.18}$$

Eq. 3.18 shows that the adsorption rate is a quadratic function of θ at a given C_0 and X. When adsorption reaches equilibrium, $d\theta/dt$ becomes zero, and θ reaches its equilibrium value (θ_e). Solving Eq. 3.18 for the equilibrium coverage fraction (θ_e) gives

$$\theta_e = \frac{k_a(q_m X + C_0) + k_d - \sqrt{\Delta_1}}{2k_a q_m X} \tag{3.19}$$

in which Δ_1 is the discriminant of Eq. 3.18 and is defined by

$$\Delta_1 = k_a^2(C_0 - q_m X)^2 + 2k_a k_d(C_0 + q_m X) + k_d^2 \tag{3.20}$$

Substitution of Eq. 3.19 into Eq. 3.13 leads to

$$\frac{d\theta}{dt} = k_1(\theta_e - \theta) + k_2(\theta_e - \theta)^2 \tag{3.21}$$

in which

$$k_1 = \sqrt{\Delta_1} \tag{3.22}$$

$$k_2 = k_a q_m X \tag{3.23}$$

Eq. 3.21 clearly shows that Langmuir kinetics is composed of a pure first-order term and a second-order term. This new expression (Eq. 3.21) of Langmuir kinetics (Eq. 3.13) for the first time shows that the empirical first- or second-order rate equation for adsorption would be a simplified case of the general Langmuir kinetics.

3.3.5.2 Sufficient and necessary conditions for reduction of Langmuir kinetics to first- and second-order rate equations

Reduction to first-order rate equation

If k_1 is much greater than $k_2(\theta_e-\theta)$, Eq. 3.21 reduces to Eq. 3.24, known as the first-order rate equation for adsorption:

$$\frac{d\theta}{dt} \approx k_1(\theta_e - \theta) \tag{3.24}$$

In fact, $k_1 >> k_2(\theta_e-\theta)$ is the sufficient and necessary condition for the Langmuir kinetics to be simplified to the first-order rate equation and it can be further expressed to

$$\theta >> \theta^* = \theta_e - \frac{k_1}{k_2} \tag{3.25}$$

In adsorption study, θ for a given C_0 is related to adsorption time (t), thus the time corresponding to θ* is designated as t*. According to Eq. 3.25, two cases can be seen: (i) if k_1/k_2 is greater than θ_e, then $\theta > \theta^*$ is always valid as θ is positive. Thus, the Langmuir kinetics can be reasonably reduced to Eq. 3.24; (ii) in case where k_1/k_2 is very close to θ_e, i.e. θ* or t* is very close to zero, θ should be greater than zero according to Eq. 3.25, indicating that the entire adsorption process with a given C_0 would be reasonably described by the first-order rate equation (Eq. 3.24).

Reduction to second-order rate equation

If $k_1 << k_2(\theta_e - \theta)$, Eq. 3.21 can be simplified to

$$\frac{d\theta}{dt} \approx k_1(\theta_e - \theta)^2 \tag{3.26}$$

which is a typical second-order rate equation for adsorption. In fact, $k_1 << k_2(\theta_e - \theta)$ is the sufficient and necessary condition for the Langmuir kinetics to be reduced to the second-order kinetics, and it can be further expressed as

$$\theta << \theta^* = \theta_e - \frac{k_1}{k_2} \tag{3.27}$$

At $k_1/k_2 << \theta_e$, θ^* is very close to θ_e, i.e. adsorption kinetics can be reasonably described by the second-order rate equation (Eq. 3.26) over the whole adsorption period.

Hybrid-order rate equation for adsorption

In addition to the above discussion, in cases where k_1/k_2 is neither close to zero nor close to θ_e, the Langmuir kinetics can not be simplified to the first-order or second-order rate equation, and adsorption needs to be described by the Langmuir kinetics (Eq. 3.21) with a varying reaction order of 1 to 2.

Calculation of θ_e and k_1/k_2

As discussed above, simplification of the Langmuir kinetics to first- or second-order rate equation is governed by the relative magnitude of θ_e over k_1/k_2. And θ_e and k_1/k_2 can be obtained through the following formulas.

At equilibrium, Eq. 3.9 becomes

$$\theta_e = \frac{C_0 - C_e}{q_m X} \tag{3.28}$$

which can be used to determine θ_e under the given experimental conditions.

When adsorption reaches its equilibrium, the equilibrium constant K_L in Langmuir isotherm (Eq. 3.2) is defined as:

$$K_L = \frac{k_a}{k_d} \tag{3.29}$$

Substitution of Eq. 3.29 into Eq. 3.19 yields

$$\theta_e = \frac{K_L(q_m X + C_0) + 1 - \sqrt{K_L^2(C_o - q_m X)^2 + 2K_L(C_0 + q_m X) + 1}}{2K_L q_m X} \tag{3.30}$$

which can be used to calculate θ_e theoretically.

According to Eqs. 3.20, 3.22 and 3.23, the k_1/k_2 can also be expressed as

$$\frac{k_1}{k_2} = \frac{\sqrt{k_a^2(C_0 - q_m X)^2 + 2k_a k_d(C_0 + q_m X) + k_d^2}}{k_a q_m X} \tag{3.31}$$

Substitution of Eq. 3.29 into Eq. 3.31yields

$$\frac{k_1}{k_2} = \frac{\sqrt{K_L^2(C_0 - q_m X)^2 + 2K_L(C_0 + q_m X) + 1}}{K_L q_m X} \tag{3.32}$$

Under given conditions, C_0 and X in Eq. 3.32 are known, and K_L and q_m are obtainable from Eq. 3.2. Thus, Eq. 3.32 can be used for calculating the ratio of k_1/k_2. Therefore the proposed sufficient and necessary conditions for simplification of Langmuir kinetics to first- and second-order rate equations as proposed in this section become applicable.

In this study, θ_e and k_1/k_2 were calculated using Eqs. 3.30 and 3.32 and their values are summarized in Table 3.5. For the verification purpose, the calculated f values in Table 3.4 are also listed in the right column in Table 3.5. It was found that for PCG, at $C_0 \leq 1000$ mg L^{-1}, k_1/k_2 was greater than θ_e (Table 3.5). As discussed earlier, in this case, the Langmuir kinetics (Eq. 3.21) can be reasonably reduced to the first-order rate equation (Eq. 3.24). Concurrently, it can be seen in Table 3.5 that for runs No. 1 to 4, the f values of the first-order rate equation were very close to those of the Langmuir kinetics, while the f values of the second-order rate equation were much lower than one. These provide statistical evidence for the simplification of the Langmuir kinetics to the first-order rate equation at $C_0 \leq 1000$ mg L^{-1}. At the initial PCG concentration of 1500 mg L^{-1}, the Langmuir kinetics cannot be simplified to the first- or second-order rate equation, and this is justified by the low f values for the first- and second-order rate equations (Table 3.5, run No. 5). The similar situations were also observed in the cases of AMP and CPC adsorption by GAC (Table 3.5).

Table 3.5 Predicted adsorption kinetics of antibiotics by GAC.

No.	Antibiotics	C_0 (mg L^{-1})	Experimental θ_e by Eq. 3.28	Theoretical θ_e by Eq. 3.30	Theoretical k_1/k_2 by Eq. 3.32	Theoretical θ_e-k_1/k_2	Predicted kinetics	f value		
								1^{st}-order	2^{nd}-order	Langmuir
1	PCG	20	0.0092	0.0092	1.01	<0	1st-order	0.999	0.0034	1.00
2	PCG	100	0.047	0.046	0.97	<0	1st-order	0.999	0.010	1.00
3	PCG	200	0.092	0.092	0.93	<0	1st-order	0.999	0.046	1.00
4	PCG	1000	0.45	0.45	0.58	<0	1st-order	0.999	0.998	1.00
5	PCG	1500	0.67	0.67	0.38	0.29	Langmuir	0.389	0.269	1.00
6	AMP	20	0.023	0.023	1.03	<0	1st-order	0.924	0.120	1.00
7	AMP	100	0.12	0.12	0.95	<0	1st-order	0.942	0.228	1.00

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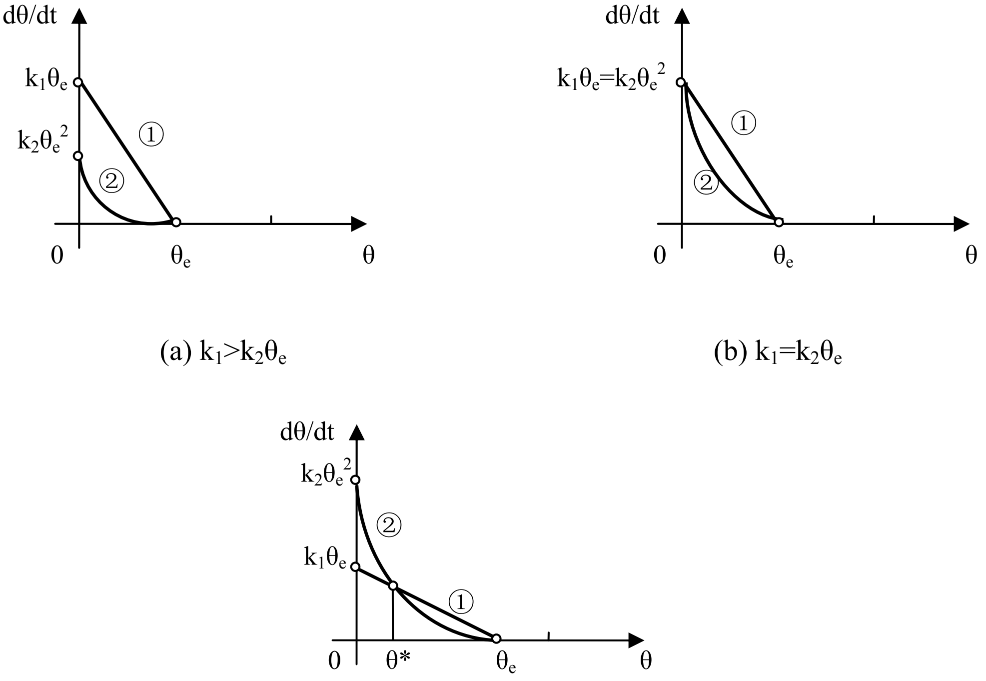
8	AMP	200	0.23	0.23	0.84	<0	1st-order	0.606	0.248	1.00
9	AMP	800	0.77	0.78	0.47	0.31	Langmuir	0.515	0.438	1.00
10	AMP	1000	0.88	0.87	0.54	0.33	Langmuir	0.606	0.392	1.00
11	CPC	10	0.055	0.059	0.96	<0	1st-order	0.995	0.582	1.00
12	CPC	20	0.11	0.12	0.90	<0	1st-order	0.663	0.127	1.00
13	CPC	40	0.23	0.23	0.78	<0	1st-order	0.986	0.190	1.00
14	CPC	60	0.35	0.35	0.67	<0	1st-order	0.913	0.179	1.00
15	CPC	80	0.46	0.46	0.56	<0	1st-order	0.673	0.601	1.00
16	CPC	100	0.52	0.58	0.45	0.13	2nd-order	0.732	0.993	1.00

3.3.5.3 A general principle for simplification of Langmuir kinetics to first- and second-order rate equations

It appears from the discussion above that the simplification of Langmuir kinetics is essentially dependent on the relative difference of "θ" and "θ_e-k_1/k_2". However, θ is a variable over the adsorption time. Therefore, a more general principle for the simplification of Langmuir kinetics to first- or second-order kinetics is highly desirable in a practical adsorption process.

As Eq. 3.21 shows, Langmuir kinetics indeed is a combination of two terms: first-order term $k_1(\theta_e-\theta)$ and the second-order term $k_2(\theta_e-\theta)^2$. It appears that the relative weights of first-order and second-order terms govern the approximation of Eq. 3.21 to the first-order or second-order kinetics for a given adsorption process. Figure 3.9 presents the plots of the overall adsorption rate over $k_1(\theta_e-\theta)$ and $k_2(\theta_e-\theta)^2$ respectively. The straight line ① represents the plot of the pure first-order rate equation, i.e. $d\theta/dt= k_1(\theta_e-\theta)$, whereas the curve ② exhibits the plot of the pure second-order rate equation, $d\theta/dt= k_2(\theta_e-\theta)^2$. Therefore, the relative contribution of the first-order and the second-order terms to the overall adsorption rate can be visualized according to the magnitude of θ. For instance, if the straight line ① is situated above the parabola ②, i.e. the value of the first-order term is bigger than that of the second-order term, as the result, Eq. 3.21 tends to approach the first-order other than to the second-order rate equation. On the contrary, if the parabola ② is above the straight line ①, meaning that the value of the second-order term is bigger than that of the first-order term, thus Eq. 3.21 can be approximated more appropriately by the second-order than first-order rate equation.



(a) $k_1>k_2\theta_e$ (b) $k_1=k_2\theta_e$

(c) $k_1<k_2\theta_e$

Figure 3.9 Plots of dθ/dt versus θ. ①: Pure first-order kinetics, $d\theta/dt=k_1(\theta_e-\theta)$; ②: Pure second-order kinetics, $d\theta/dt=k_2(\theta_e-\theta)^2$.

According to the position of the intersection point of curves ① and ② in Figure 3.9, two situations can be differentiated:

(1) Figure 3.9 (a) and (b) show a case where the straight line ① is always above the curve ② in the range of $0<\theta<\theta_e$. The corresponding algebraic condition thus can be translated into $k_1\geq k_2\theta_e$, indicating that the contribution of the second-order term over the first-order term would be minor.

(2) Figure 3.9 (c) shows that the straight line ① intersects with the curve ② at a critical θ value (θ*) in the range of $0<\theta<\theta_e$. The corresponding algebraic condition can be described as $k_1<k_2\theta_e$, and θ* is equal to (θ_e-k_1/k_2) (referring to Eq. 3.25). Under this circumstance, two subsituations are further identified: (i) if $\theta<\theta^*$, the curve ② is above the straight line ①, suggesting that Eq. 3.21 would approach the

second-order kinetics; (ii) if $\theta > \theta^*$, the straight line ① is above the curve ②, meaning that Eq.3.21 would be reasonably approximated by the first-order instead of the second-order rate equation. As the term θ^* may also be translated to the corresponding time (t^*), the above two subconditions, e.g. $\theta < \theta^*$ and $\theta > \theta^*$ can be expressed further in terms of t^*. For instance, at $0 < t < t^*$, Eq. 3.21 would approach the second-order rate equation, whereas at $t^* < t < t_e$, it would be close to the first-order rather than second-order kinetics.

Eq. 3.21 shows that the relative value of the term k_1 against the term $k_2\theta_e$ indeed governs its simplification. For simplification of Eq. 3.21, the basic idea is to solve the inequality $k_1 \geq k_2\theta_e$ or $k_1 < k_2\theta_e$. It appears from Eqs. 3.22 and 3.23 that that the solutions of the inequality $k_1 \geq k_2\theta_e$ or $k_1 < k_2\theta_e$ are only dependent on k_a, k_d, q_m, X and C_0. To solve inequality $k_1 \geq k_2\theta_e$ or $k_1 < k_2\theta_e$, the corresponding equivalent inequality with obvious solutions is constructed in this study.

In order to compare k_1 and $k_2\theta_e$, a function R is defined as follows:

$$R = k_1 - k_2\theta_e \tag{3.33}$$

Inserting Eqs. 3.22 and 3.23 into Eq. 3.33 gives

$$R = 1.5\sqrt{\Delta_1} - \frac{k_a(q_m X + C_0) + k_d}{2} \tag{3.34}$$

Further let $A = 1.5\sqrt{\Delta_1}$ and $B = \dfrac{k_a(q_m X + C_0) + k_d}{2}$ (3.35)

Therefore,

$$k_1 - k_2\theta_e \geq 0(\text{or} < 0) \Leftrightarrow R \geq 0(\text{or} < 0) \Leftrightarrow A - B \geq 0(\text{or} < 0) \tag{3.36}$$

Since A, B and k_d^2 are all positives, thus

$$A - B \geq 0(\text{or} < 0) \Leftrightarrow A^2 - B^2 \geq 0(\text{or} < 0) \Leftrightarrow \frac{A^2 - B^2}{k_d^2} \geq 0(\text{or} < 0) \tag{3.37}$$

Therefore, the function G is constructed in a way such that

$$G = \frac{A^2 - B^2}{k_d^2} = \frac{(1.5\sqrt{\Delta_1})^2 - [\frac{k_a(q_m X + C_0) + k_d}{2}]^2}{k_d^2} \tag{3.38}$$

$G \geq 0$ or $G<0$ is the equivalent inequality to $k_1 \geq k_2\theta_e$ or $k_1<k_2\theta_e$, nevertheless solving $G \geq 0$ or $G<0$ is much easier than solving $k_1 \geq k_2\theta_e$ or $k_1<k_2\theta_e$. Thus, solutions for inequality $G \geq 0$ or $G<0$ are the solutions of the original inequality $k_1 \geq k_2\theta_e$ or $k_1<k_2\theta_e$.

It should be noted that Eq. 3.38 has the unique features of that (i) $G>0$ is equivalent to $k_1>k_2\theta_e$; (ii) $G<0$ is equivalent to $k_1<k_2\theta_e$, and (iii) $G=0$ has the same solution as $k_1=k_2\theta_e$. Replacing Δ_1 by Eq. 3.20, Eq. 3.38 can be further arranged to

$$G = 2K_L{}^2C_0{}^2 - (5K_L{}^2q_mX - 4K_L)C_0 + 2K_L{}^2q_m^2X^2 + 4K_Lq_mX + 2 \qquad (3.39)$$

in which K_L is the Langmuir adsorption constant defined by the Langmuir isotherm, $K_L=k_a/k_d$. At a given X, Eq.3.39 indeed represents a quadratic function of C_0 only, and its discriminant (Δ_2) is given by

$$\Delta_2 = 9K_L{}^3q_mX(K_Lq_mX - 8) \qquad (3.40)$$

The following three different scenarios thus can be generated according to Eq. 3.40.

Scenario 1: if $K_Lq_mX<8$, Δ_2 is smaller than 0, suggesting that there is no real root for C_0 in Eq. 3.39 so that $G>0$, i.e. $k_1>k_2\theta_e$ (Figure 3.9 (a)). In this case, no matter what C_0 is, Eq. 3.21 would be much closer to the first-order than to the second-order rate equation.

Scenario 2: if $K_Lq_mX=8$, $\Delta_2=0$, meaning that there is one real root only for C_0 in Eq. 3.39. In this situation, $G \geq 0$, i.e. $k_1 \geq k_2\theta_e$ (Figure 3.9 (a) and (b)). Similar to scenario 1, Eq.3.21 would be much closer to the first-order than to the second-order rate equation.

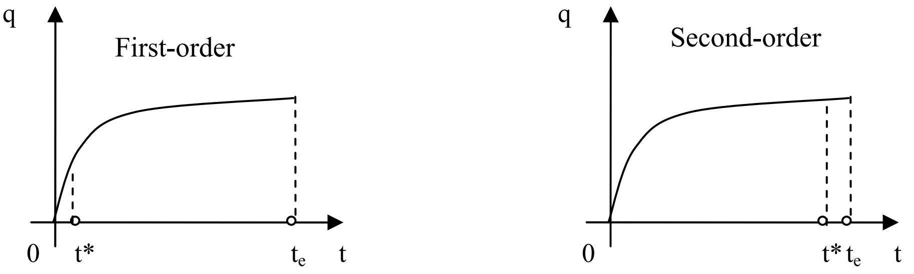
Scenario 3: if $K_Lq_mX>8$, Δ_2 is greater than 0. Thus, the following two real roots of C_0 can be derived from Eq.3.39 at $G=0$:

$$C_{0,1} = \frac{5K_L^2 q_m X - 4K_L - \sqrt{\Delta_2}}{4K_L^2} \tag{3.41}$$

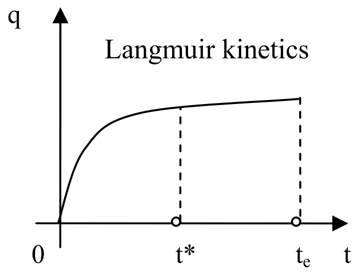
$$C_{0,2} = \frac{5K_L^2 q_m X - 4K_L + \sqrt{\Delta_2}}{4K_L^2} \tag{3.42}$$

According to $C_{0,1}$ and $C_{0,2}$, two special situations are accounted for. (i) If $C_0 \leq C_{0,1}$ or $C_0 \geq C_{0,2}$, then $G \geq 0$, i.e. $k_1 \geq k_2\theta_e$ (Figure 3.9 (a) and (b)). This implies that the Langmuir kinetics is closer to the first-order than to the second-order rate equation. (ii) If $C_{0,1} < C_0 < C_{0,2}$, then G is smaller than 0, i.e. $k_1 < k_2\theta_e$ (Figure 3.9 (c)). In this case, the Langmuir kinetics cannot be simply simplified to the first-order or second-order rate equation for the entire period of adsorption time, and the reaction order indeed varies with adsorption time. For instance, at $0 < t < t^*$, the second-order is dominant over the first-order, whereas at $t > t^*$, the first-order will overtake the second-order.

As discussed earlier, in cases where $C_{0,1} < C_0 < C_{0,2}$, the Langmuir kinetics would be approximated by the second-order rate equation at $t < t^*$; while at $t > t^*$, it tends to the first-order one. This implies that the proportion of t* over the whole effective adsorption time defined as the time required to reach equilibrium (t_e), indeed governs the overall simplification of the Langmuir kinetics to the first-order or second-order rate equation at $C_{0,1} < C_0 < C_{0,2}$. If the t^*/t_e ratio is close to zero, the overall adsorption kinetics is much closer to the first-order than the second-order (Figure 3.10 (a)), while in case where the t^*/t_e ratio approaches to one, the Langmuir kinetics would reduce to the second-order instead of the first-order rate equation (Figure 3.10 (b)). However, if the t^*/t_e ratio is neither close to zero nor one, adsorption kinetics would be better described by the hybrid-order Langmuir kinetics rather than the first- or second-order rate equation (Figure 3.10 (c)).



(a) t^*/t_e close to 0 (b) t^*/t_e close to 1



(c) t^*/t_e neither close to 0 nor 1

Figure 3.10 Illustration of t^*/t_e-dependent simplification of Langmuir kinetics at $C_{0,1}<C_0<C_{0,2}$.

According to the above theoretical analyses, a general principle for simplification of the Langmuir kinetics is summarized in Figure 3.11, and it is related to the respective magnitudes of the terms K_Lq_mX, $C_{0,1}$, $C_{0,2}$ and t^*/t_e, which represent the influence of adsorbent, adsorbate and adsorption time on the whole process kinetics, respectively. To verify the proposed simplification principle, experimental data were used to calculate K_Lq_mX, $C_{0,1}$, $C_{0,2}$ and t^*/t_e (Table 3.6).

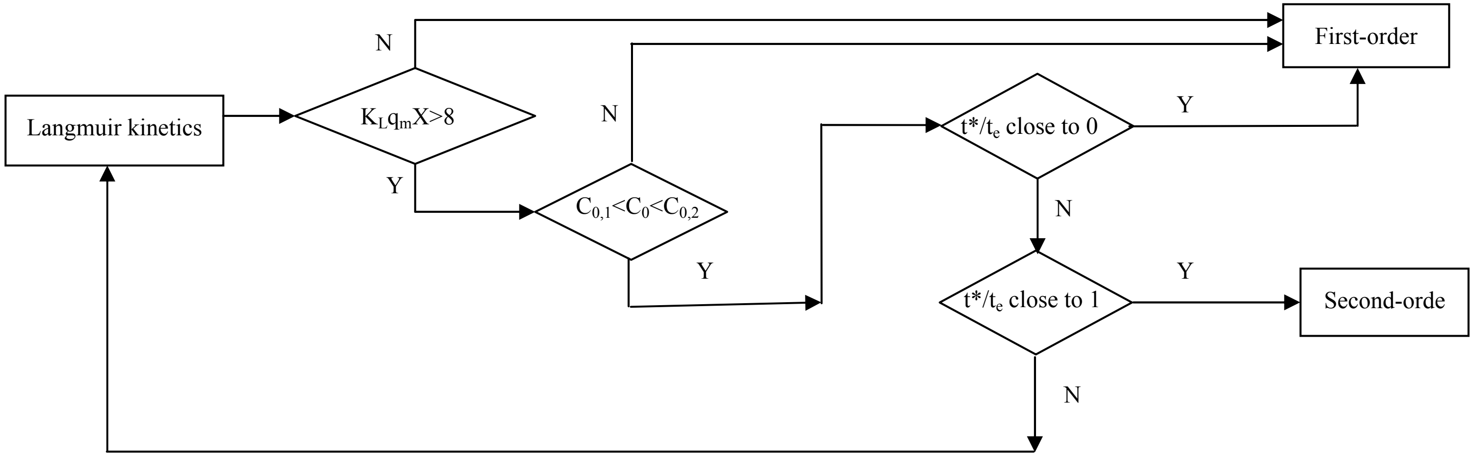


Figure 3.11 A general principle for simplifying the Langmuir kinetics to first- or second-order rate equation.

Table 3.6 Boundary conditions for simplification of the Langmuir kinetics at various antibiotic concentrations and the fixed GAC dosage of 5 g L^{-1}, 25°C.

No.	Antibiotics	C_0 (mg L^{-1})	K_Lq_mX	$C_{0,1}$ (mg L^{-1})	$C_{0,2}$ (mg L^{-1})	t*(h)
1	PCG	20	59.6	1144	4126	-
2	PCG	100	59.6	1144	4126	-
3	PCG	200	59.6	1144	4126	-
4	PCG	1000	59.6	1144	4126	-
5	PCG	1500	59.6	1144	4126	5
6	AMP	20	18.6	518	1446	-
7	AMP	100	18.6	518	1446	-
8	AMP	200	18.6	518	1446	-
9	AMP	800	18.6	518	1446	7
10	AMP	1000	18.6	518	1446	2.8
11	CPC	10	78.0	89	328	-
12	CPC	20	78.0	89	328	-
13	CPC	40	78.0	89	328	-
14	CPC	60	78.0	89	328	-
15	CPC	80	78.0	89	328	-
16	CPC	100	78.0	89	328	22

In this study, the values of K_Lq_mX were estimated as 59.6, 18.6 and 78.0 for the adsorption of PCG, AMP and CPC, respectively (Table 3.6), which are much higher than the critical values of 8, meaning that the adsorption kinetics of these antibiotics by GAC would highly depend on C_0 and t as elaborated earlier. Comparison of C_0, $C_{0,1}$ and $C_{0,2}$ (Table 3.6) shows that C_0 was smaller than $C_{0,1}$ and $C_{0,2}$ in the most experiments carried out in this study. This in turn implies that the Langmuir kinetics would be approximated by the first-order rate equation instead of the second-order one, e.g. the experiments No.1-4: C_0=20 to 1000 mg L^{-1} for PCG; No. 6-8: C_0=20 to 200 mg L^{-1} for AMP; No. 11-15: C_0=10 to 80 mg L^{-1} for CPC. This is further confirmed by the error analysis in terms of f given in Table 3.5.

For the experiment No. 5 (C_0=1500 mg L^{-1} for PCG), No. 8 and 9 (C_0=800 mg L^{-1}, 1000 mg L^{-1} for AMP) and No.16 (C_0= mg L^{-1} for CPC), C_0 was found to be fallen in between $C_{0,1}$ and $C_{0,2}$. According to the proposed "principle for simplification of the Langmuir kinetics" (Figure 3.9), the reaction order should vary from the second-order to first-order over time in this case. The estimated t* values are shown in Table 3.6. As discussed earlier, at 0<t<t*, the Langmuir kinetics would be approximated by the second-order, while at t>t*, it should more approach the first-order, implying that the proportion of t* to the whole time period of the adsorption experiments governs the overall approximation of the adsorption kinetics, either to first-order or to second-order. In this study, all the adsorption experiments lasted for at least 24 hours at least. The comparison of t* with the whole experimental time revealed that only for the experiment No. 16 (C_0=100 mg L^{-1} for CPC), t* had a value of 22 hours which accounts for 91.7% of the whole experimental time (24 hours). As the result, only in this case, the Langmuir kinetics could be simplified to the second-order kinetics. On the contrary, in the experiments No. 5, 8 and 9 (Table 3.6), the second-order dominating time is less than 10% of the whole experimental period, i.e. the overall adsorption kinetics can be reduced to the first-order kinetics. These are also confirmed by the error analysis in terms of f (Table 3.5).

In order to further verify the simplification principle proposed in this study, experimental data from the literature were also used and results are summarized in Table 3.7. It was found that the proposed simplification principle is valid for all the cases presented in Table 3.7.

Table 3.7 Verification of the proposed simplification principle using the literature data.

Adsorbate	Adsorbent	K_Lq_mX	$C_{0,1}$ (mg L^{-1})	$C_{0,2}$ (mg L^{-1})	C_0 (mg L^{-1})	t^*/t_e (%)	Criteria	Predicted kinetics	f value			References
									1st- order	2nd- order	Langmuir	
Cr (VI)	Kaolinite	64.4	12.4	44.9	50	-	$C_0>C_{0,2}$	1st-order	0.574	0.032	1.00	Bhattacharyya and SenGupta 2006
Cr (VI)	Acid-activated kaolinite	78.6	14.6	54.2	50	4.7	$t^*/t_e\approx 0$	1st-order	0.175	0.077	1.00	Bhattacharyya and SenGupta 2006
Cr (VI)	ZrO-kaolinite	62	11.6	42.2	50	-	$C_0>C_{0,2}$	1st-order	0.999	0.029	1.00	Bhattacharyya and SenGupta 2006
Cr (VI)	TBA-kaolinite	59.4	11.4	40.9	50	-	$C_0>C_{0,2}$	1st-order	0.999	0.033	1.00	Bhattacharyya and SenGupta 2006
Cs (I)	Aluminum -pillared montmorillonite	4.132	-	-	-	-	$K_Lq_mX<8$	1st-order	0.999	0.288	1.00	Karamanis and Assimakopoulos 2007
Zn (II)	Natural sorbents	4.488	-	-	-	-	$K_Lq_mX<8$	1st-order	0.999	0.418	1.00	Al-Degs et al. 2006

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Cr (VI)	Mg-Al-CO_3 hydrotalcite	12.815	18.56	38.82	10	-	$C_0<C_{0,1}$	1st-order	0.999	0.029	1.00	Lazaridis and Asouhidou 2003
Cr (VI)	Mg-Al-CO_3 hydrotalcite	12.815	18.56	38.82	50	-	$C_0>C_{0,2}$	1st-order	0.999	0.040	1.00	Lazaridis and Asouhidou 2003
Cr (VI)	Mg-Al-CO_3 hydrotalcite	12.815	18.56	38.82	100	-	$C_0>C_{0,2}$	1st-order	0．246	0．022	1.00	Lazaridis and Asouhidou 2003
As(V)	Ce-Fe	46.0	2.33	8.02	1.0	-	$C_0<C_{0,1}$	1st-order	0.990	0.234	1.00	Zhang et al. 2003
Ni(II)	algae	11.25	947.5	1955	50	-	$C_0<C_{0,1}$	1st-order	0.858	0.264	1.00	Kalyani et al. 2004
Pb(II)	Kaolinitic clay	100.0	49.8	186.1	5	-	$C_0<C_{0,1}$	1st-order	0.351	0.271	1.00	Orumwense 1996
Pb(II)	Clinoptilolite	22.5	110.6	331.4	15	-	$C_0<C_{0,1}$	1st-order	0.999	0.152	1.00	Gunay et al. 2007
Pb(II)	Clinoptilolite	22.5	110.6	331.4	30	-	$C_0<C_{0,1}$	1st-order	0.999	0.079	1.00	Gunay et al. 2007
Fluoride	Collagen fiber	480.5	20.1	82.5	95	-	$C_0>C_{0,2}$	1st-order	0.998	0.153	1.00	Liao and Shi 2005
Fluoride	Iron(III)-zirconium(IV) hybrid oxide	109.0	7.4	28.0	5	-	$C_0<C_{0,1}$	1st-order	0.439	0.1649	1.00	Biswas et al. 2007

(to be continued)

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Cu(II)	H_3PO_4-activated rubber wood sawdust	49.7	15.7	54.6	20	96.66	$t^*/t_e \approx 1$	2nd-order	0.337	0.999	1.00	Kalavathy et al. 2005
Methylene blue	Cedar sawdust	216.4	36.3	140.9	40	99.99	$t^*/t_e \approx 1$	2nd-order	0.269	0.819	1.00	(Hamdaoui 2006)
p-nitrophenol	Activated carbon fiber	222.3	391.6	1524.5	1000	99.60	$t^*/t_e \approx 1$	2nd-order	0.151	0.998	1.00	Tang et al. 2007
Cu(II)	H_3PO_4-activated rubber wood sawdust	49.7	15.7	54.6	40	96.66	$t^*/t_e \approx 1$	2nd-order	0.234	0.999	1.00	Kalavathy et al. 2005
Fluoride	Iron(III)-zirconium(IV) hybrid oxide	109.0	7.4	28.0	12.5	75	t^*/t_e neither close to 0 nor to 1	Langmuir kinetics	0.532	0.683	1.00	Biswas et al. 2007
Fluoride	Iron(III)-zirconium(IV) hybrid oxide	109.0	7.4	28.0	25	68	t^*/t_e neither close to 0 nor to 1	Langmuir kinetics	0.639	0.715	1.00	Biswas et al. 2007

3.4 DISCUSSION

For adsorption of PCG, AMP and CPC by GAC, Langmuir isotherm was found to offer the best prediction of the equilibrium data at all the temperatures studied, and the maximum adsorption capacity (q_m) derived from Langmuir isotherm was 427.3 mg g^{-1} (1.2 mmol g^{-1}) for PCG, 164.2 mg g^{-1} (0.5 mmol g^{-1}) for AMP and 33.67 mg g^{-1} (0.1 mmol g^{-1}) for CPC at 25°C, respectively. Dutta et al. (1997a, b) also investigated the adsorption of various β-lactam antibiotics, such as 6-aminopenillanic acid, 7-aminocephalosporanic acid, cephalexin and cefadroxyl by activated carbon, and found that Langmuir isotherm could best fit to the equilibrium data for those antibiotics, and the values of the maximum adsorption capacity ranged from 0.2 to 2.8 mmol g^{-1} at 25°C. Recently, adsorption of other types of antibiotic by GAC was studied as well, such as nitroimidazole (Rivera-Utrilla et al. 2009), trimethoprim (Kim et al. 2010) and sulfamethoxazole (Çalişkan and Göktürk 2010). This and previous studies indeed show that GAC has a substantial adsorption capacity of various types of antibiotics.

The FTIR spectra (Figure 3.2) and thermodynamic analysis indicated a chemisorption mechanism of GAC adsorption of PCG, AMP and CPC. In the FTIR spectra, the carbonyl group and hydronitrogen linkage in the β-lactam ring occurred as a broad band centered at 1700 cm^{-1} and 3480 cm^{-1} respectively, and a remarkable change in the fingerprint region of FTIR spectra was observed after the binding of antibiotic onto the surface of GAC. Moreover, the estimated values of adsorption enthalpy $\Delta H°$ were around 60 kJ mol^{-1} for the antibiotics studied. Generally, if value of the adsorption enthalpy is smaller than 20 kJ mol^{-1}, adsorption can be reasonablely assumed to be physisorption, whereas chemisorption would be dominant adsorption mechanism at the adsorption enthalpy greater than 40 kJ mol^{-1}

(Bansal and Goyal 2005). The magnitude of the ΔH° values for PCG, AMP and CPC falls into the chemisorption range, suggesting that adsorption of PCG, AMP and CPC by GAC was a chemical-dominant process. However, it should be pointed out that the thermodynamic mechanisms of adsorption would be dependent on the chemical structures of various antibiotics. For example, ΔH° values reported in the adsorption of 7-aminocephalosporanic acid, cephalexin, cefadroxyl and 6-aminopenillanic acid by activated carbon ranged from 17 to 46 kJ mol^{-1} (Dutta et al. 1999), implying a physical-chemical mixed mechanism. In addition, adsorption of sulfamethoxazole by GAC was even found a physical nature (Çalişkan and Göktürk 2010).

A new transformed Langmuir kinetic equation (Eq. 3.21) was developed in this study, and for the first time it was clearly demonstrated that the first- and second-order rate equations are the simplified forms of the general Langmuir kinetics. Rudzinski and Plazinski (2006) also attempted to offer theoretical basis of the first- and second-order kinetic equations for adsorption by introducing the statistical rate theory of interfacial transport, and the following central equation was obtained:

$$\frac{d\theta}{dt} = \alpha_1(\theta_e - \theta) + \alpha_2(\theta_e - \theta)^3 + \alpha_3(\theta_e - \theta)^5 + \ldots \qquad (3.43)$$

in which α_1, α_2 and α_3 are constant coefficients. As compared to the transformed Langmuir kinetics (Eq. 3.21), it should be realized that the second-order term is missing in Eq. 3.43. Rudzinski and Plazinski (2006) thought that if the second and third terms in Eq. 3.43 are much smaller than the first one, Eq. 3.43 can be reduced to the first-order kinetic model. Because of the complexity of Eq. 3.43, no initial adsorbate-based boundary conditions were given for simplification of Eq. 3.43 to the first-order rate equation. On the other hand, it was further proposed by Rudzinski and Plazinski (2006) if the third term onwards in Eq. 3.43 is negligible compared to the first two terms, Eq. 3.43 can be approximated by

$$\frac{d\theta}{dt} = \alpha_1(\theta_e - \theta) + \alpha_2(\theta_e - \theta)^3 \tag{3.44}$$

Obviously, Eq. 3.44 shows a hybrid-order between the first and the third power, implying that the second-order kinetic equation cannot be naturally obtained from Eq. 3.44. Rudzinski and Plazinski (2006) thought that the second-order kinetic equation might simulate such a hybrid behavior, and they believed that this would explain the theoretical background of the second-order rate equation for adsorption. Nevertheless, mathematically Eq. 3.44 cannot be readily attributed to a second-order rate equation, and this in turn implies that the statistical rate theory-based approach may not provide the theoretical background of the second-order kinetic equation for adsorption. In this regard, as shown above, the transformed Langmuir kinetics (Eq. 3.21) and the geometric method proposed in this study might offer in-depth insights into the possible theoretical origins of the widely used first- and second-order rate equations for adsorption.

Starting from the Langmuir kinetics (Eq. 3.13), Azizian (2004) also looked into simplification of the Langmuir kinetics to the first- and second-order rate equations for adsorption. Azizian (2004) thought that change in concentration of solute in solution can be described in a way such that:

$$C = C_0 - \beta\theta \tag{3.45}$$

in which β is a constant under given conditions. Comparison of Eq. 3.45 with Eq. 3.10 suggests that β is indeed equal to $q_m X$ if the volumetric concentration of adsorbate is used. Substitution of Eq. 3.45 into Eq. 3.13 yields the following expression:

$$\frac{d\theta}{dt} = k_a(C_0 - \beta\theta)(1-\theta) - k_d\theta \tag{3.46}$$

Different from the transformed Langmuir kinetics developed in this study (Eq. 3.21), Azizian (2004) used Eq. 3.46 as a basis to explain the theoretical origins of the first-and second-order rate equations. Azizian (2004) thought that the first-order kinetic equation can be obtained from Eq. 3.46 by assuming $C_0 >> \beta\theta$, and concluded that "the sorption process obeys first-order kinetics at high initial concentration of solute". However, it should be realized that θ is not constant and varies from 0 to θ_e in the course of adsorption experiment. This indicates that validation of the assumption of $C_0 >> \beta\theta$ largely depends on the magnitude of the variable θ. Furthermore, at $C_0 >> \beta\theta$, Eq. 3.45 reduces to $C \approx C_0$, indicating that change in the solute concentration during adsorption can be neglected, i.e. the assumption of $C_0 >> \beta\theta$ is valid only for a system in which adsorption of solute is negligible. Obviously, this is not the actual case encountered in adsorption studies. In addition, under the assumption of $C_0 >> \beta\theta$, according to Azizian (2004), Eq. 3.46 is simplified to

$$\frac{d\theta}{dt} \approx k_a C_0 \text{-} (k_a C_0 + k_d)\theta \qquad (3.47)$$

When adsorption reaches its equilibrium, $d\theta/dt=0$ and $\theta=\theta_e$ and Eq. 3.47 thus becomes

$$\theta_e \approx \frac{K_L C_0}{1 + K_L C_0} \qquad (3.48)$$

Compared with the Langmuir isotherm (Eq. 3.3), the assumption of $C_0 >> \beta\theta$ eventually leads to $C_0 \approx C_e$, this can be found only in an adsorption system in which adsorption is negligible as discussed above. Consequently, the Langmuir kinetics would not be readily simplified to the first-order rate equation by the simple assumption of $C_0 >> \beta\theta$. In fact, it should be realized that $C_0 >> \beta\theta$ indeed is not the sufficient and necessary condition for simplification of the Langmuir kinetics to the first-order rate equation.

Similar to the integrated Langmuir kinetics given by Novak and Adriano (1975), Azizian (2004) expressed the integrated Langmuir kinetics as follows:

$$\theta = \frac{\xi\gamma(e^{\lambda t} - 1)}{2a(\xi - \gamma e^{\lambda t})} \tag{3.49}$$

in which λ, ξ, γ and a are all constants under given conditions. According to Azizian (2004), if the term λt in Eq. 3.49 is very small, then the term exp(λt) can be approximated by

$$e^{\lambda t} \approx 1 + \lambda t \tag{3.50}$$

and Eq. 3.49 reduces to

$$\theta \approx -\frac{\theta_e \gamma \lambda t}{2\lambda\text{-}\gamma\lambda t} \tag{3.51}$$

In Azizin's approach, the constant λ is given by

$$\lambda = k_a\sqrt{(\beta + C_o + 1/K_L)^2 - 4C_o\beta} \tag{3.52}$$

Since $\theta = q/q_m$ and $\theta_e = q_e/q_m$, Eq. 3.51 can be rearranged to

$$\frac{t}{q} = \frac{1}{k_2^{'} q_e^2} + \frac{1}{q_e} t \tag{3.53}$$

that is the integrated form of the second-order rate equation. Azizian (2004) thus concluded that "the sorption process obeys the second-order model at lower initial concentration of solute". It is not clear about how small λt is necessarily related to lower initial concentration of solute. In fact, Eq. 3.52 suggests that λ is a constant under given conditions. As the result, the assumption of small λt can be naturally translated into another expression of that t should be very small. This implies that the second-order rate equation derived from Azizian's approach would be valid only for initial stage of adsorption at which t can be reasonably assumed to be small. Similar to θ, t is a variable instead of constant. Theoretically, t may vary in a very large time scale in the course of adsorption experiment (e.g. from zero to infinite), depending on the nature of adsorption process. Mathematically, a small λ also cannot naturally ensure a small λt. Consequently, the assumption of small λt might not lead to the conclusion of "the sorption process obeys the second-order model at lower initial concentration of solute" by Azizian (2004).

3.5 SUMMARY

This study shows that GAC is an effective adsorbent for the removal of β-lactam antibiotics, such as PCG, AMP and CPC. It was found that the antibiotics would first adsorb into the large and medium pores of the GAC, and were further accumulated in the micropores. The FTIR analysis indicated that the β-lactam ring of these three antibiotics could form the strong chemical bonds (e.g. C=O and N-H) with functional groups on the GAC surface. The adsorption capacity of antibiotics by GAC was in the order of PCG>AMP>CPC, e.g. their respective maximum adsorption capacity was 427.3 mg g^{-1}, 164.2 mg g^{-1} and 33.67 mg g^{-1} at 25°C. The equilibrium data can be best represented by the Langmuir isotherm. The positive enthalpy value confirms the endothermic nature of the adsorption process, whereas the magnitude of enthalpy suggests that the adsorption of these antibiotics by GAC was chemisorption-predominant.

This study for the first time showed that the widely used empirical first- and second-order rate equations for adsorption can be simplified from Langmuir kinetics, i.e. first- and second-order rate equations would be two special cases of Langmuir kinetics. Based on a new transformed Langmuir kinetic equation, the sufficient and necessary conditions for simplification of Langmuir kinetics to first- or second- order rate equations were proposed. Furthermore, a general principle for the simplification of Langmuir kinetics for adsorption was developed and verified. This study for the first time provides a scientific explanation for the possible theoretical origins of the empirical first- and second-order rate equations for adsorption.

CHAPTER 4

ENHANCED REMOVAL OF AMPICILLIN BY COMBINED ADSORPTION AND BIODEGRADATION

4.1 INTRODUCTION

As discussed in Chapter 3, granular activated carbon (GAC) has a substantial adsorptive capacity for the β-lactam type of antibiotics. However, antibiotics are only transferred from the liquid phase to GAC without chemical or biological breakup of their molecular structure during adsorption. On the other hand, GAC would be exhausted fast in view of the large amount of wastewater to be handled in a full-scale installation. As such, the basic idea of this study is to combine adsorption and biological degradation of antibiotics into one system, i.e. a biofilm-covered GAC (BGAC) system, in which bio-regeneration could be expected. Although BGAC has been reported for the removal of various types of organic pollutants from wastewater (Fan et al. 1987; Wilderer et al. 2000; Ong et al. 2008b; Mello et al. 2010), so far little attention has been given to the removal of antibitoics by BGAC. Therefore, this study aimed to investigate the feasibility of BGAC for the removal of ampicillin (AMP), a representative of the β-lactam antibiotics, and its possible removal pathways in the continuous airlift bioreactors.

4.2 MATERIALS AND METHODS

4.2.1 Biofilm Reactor Set-up and Operation

Three identical airlift reactors, namely R1, R2 and R3, were used (Figure 4.1). GAC from Calgon Carbon Corporation, USA, as described in Chapter 3, was used as carrier for biofilm growth. The dose of GAC was fixed at 50 g L^{-1} for all the three reactors. Activated sludge taken from a local municipal wastewater treatment plant was acclimated with a synthetic wastewater as described below in a sequencing batch reactor (SBR) for two weeks. The acclimated sludge was then seeded to R1 to R3 at the biomass concentration of 6800 mg dry weight L^{-1}. The incubation of R1 to R3 lasted for three days. Afterwards, all the suspended sludge was washed out. R1 to R3 were continuously operated at a hydraulic retention time (HRT) of 8 hours and an air flow of 4.2 L min^{-1}. All the experiments were conducted at 25°C, and the physical properties of R1 to R3 were summarized in Table 4.1.

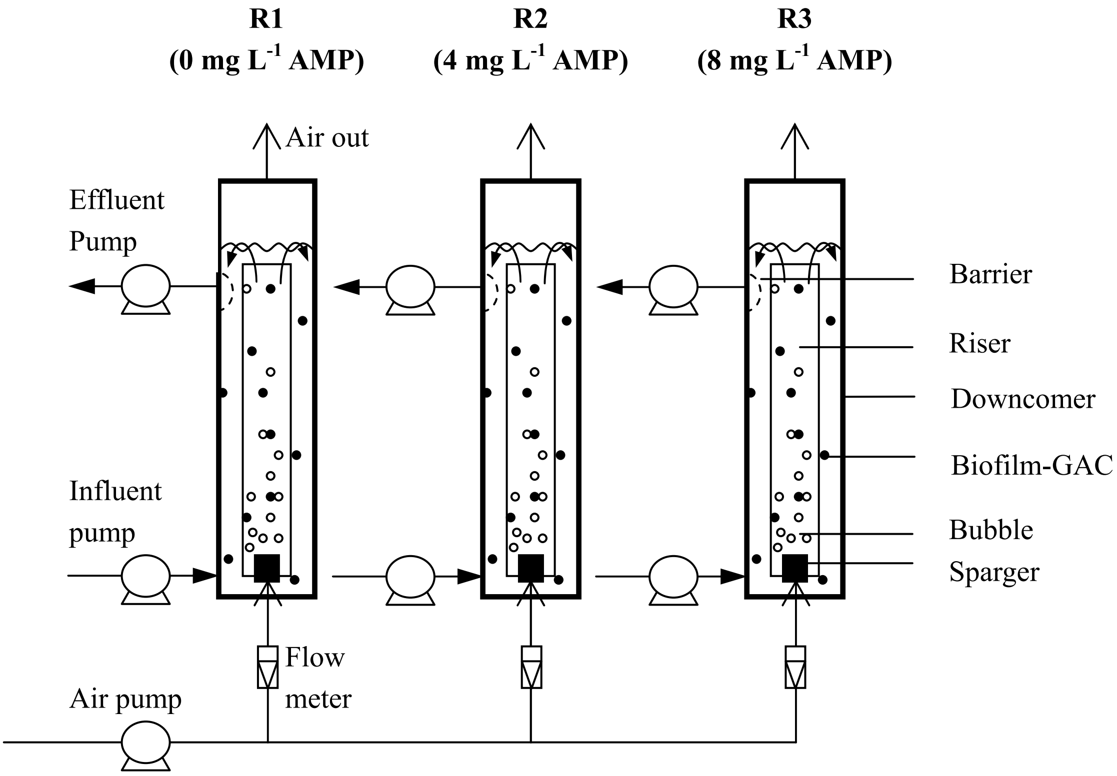


Figure 4.1 Schematic diagram of R1 to R3.

Table 4.1 Physical properties of R1 to R3.

Parameter	Value
Reactor total volume (L)	7
Reactor working volume (L)	4.5
Riser height (m)	0.5
Riser internal diameter (mm)	56
Riser external diameter (mm)	60
Downcomer internal diameter (mm)	100
Riser to bottom distance (mm)	10
HRT (h)	8
Influent or effluent flow rate (L h^{-1})	0.45
Superficial gas velocity (cm s^{-1})	3

Adopted from the previous study on biofilms in an airlift reactor (Tijhuis et al. 1994), the synthetic wastewater used in this study was composed of sodium acetate 1800 mg L^{-1}, NH_4Cl 175 mg L^{-1}, K_2HPO_4 45 mg L^{-1}, $FeSO_4{\cdot}7H_2O$ 10 mg L^{-1}, $CaCl_2{\cdot}2H_2O$ 30 mg L^{-1}, $MgSO_4{\cdot}7H_2O$ 12.5 mg L^{-1}, which a simulated municipal wastewater. The pH in each reactor was maintained at 7.0 to 7.5 by phosphate buffer (Gjaltema et al. 1995). AMP from Sigma-Aldrich Pte Ltd, Singapore, was used as the model antibiotic. R1 which was free of AMP served as the control. According to the actual AMP concentration (3.5 mg L^{-1}) found in a real pharmaceutical wastewater (Zhou et al. 2006), R2 and R3 were supplemented with 4 and 8 mg L^{-1} AMP, respectively. The superficial air velocity was fixed at 3 cm s^{-1} to maintain carriers suspended homogeneously in the airlift reactor.

4.2.2 Batch Experiments

In order to evaluate the removal potential of AMP by BGAC, a series of batch experiments were carried out with BGAC sampled from R2 and R3 on different operation days. For this purpose, 5 grams of BGAC gently rinsed with deionized water, was transferred into 100 mL of the above-mentioned synthetic wastewater, while supplemented with 4 and 8 mg L^{-1} AMP, respectively. Air was purged through a diffuser located at the bottom of the test bottle. Changes of AMP concentrations in the course of batch experiments were determined by HPLC. The temperature for all batch experiments was controlled at 25°C.

4.2.3 Analytical Methods

The fixed biomass on GAC was quantified using the method of Liu and Capdeville (1996). BGAC particles were submerged into 20 mL of 1 N NaOH solution at 80°C for 30 minutes, then received 1minute of sonification at 100 W, and followed by 30 seconds of vortex to further homogenize the detached biomass. The quantity of the detached biomass was further determined in terms of COD according to standard method (APHA 1998).

The morphology of the biofilms on GAC was observed by scanning electron microscopy (SEM) (Zeiss EVO 50, Germany). Immobilized bacterial cells on GAC were stained with 10 μg mL^{-1} 4'6-diamidino-2-phenylindole (DAPI, Sigma, Singapore) and observed with epifluorescence microscope (BX 60, Olympus, Japan). The distribution of the microorganisms on the GAC was analyzed using confocal laser scanning microscopy (CLSM) (FV300, Olympus, Japan). For this, GAC with biofilms was stained using the Live/Dead *Bac*Light Bacterial Viability Kit (Molecular Probes, USA), which contained the SYTO 9 green-fluorescent nucleic acid stain and the PI red-fluorescent nucleic acid stain. In general, the SYTO 9 labels all bacteria in a population, while PI can only stain the DNA of lysed cells. The staining was done according to in the manufacturer’s product information. The wavelengths of excitation/emission were 480/500 nm for SYTO 9 and 490/635 nm for PI, respectively.

Extracellular polymeric substances (EPS) of the biofilms were extracted by cold aqueous extraction techniques (Liu and Fang 2002). In this method, biofilms were scraped from GAC and centrifuged for 10 minutes at 4000 rpm. Supernatant was then decanted and the settled pellets were resuspended in 10 mL deionized water. Chemical extractants were added into the tube in the sequence of formaldehyde (12 μL; at 4 °C for 1 hour) and NaOH (1 N, 800μL; at 4 °C for 3 hours). After the extraction, the mixture was centrifuged at 13200 rpm for 20 minutes and the supernatant was collected for further analyses of extracellular polysaccharides (PS) and extracellular proteins (PN). The PS and PN were finally quantified by the methods of Dubois et al. (1956) and BCA protein assay kit (Sigma-Aldrich Pte Ltd, Singapore), respectively.

A respirometer (MicroOxymax, Columbus, USA) was used to study respective microbial activity of the biofilms developed in R1 to R3. This respirometer is equipped with the online oxygen probe, which allows determination of the oxygen utilization. The BGAC taken from R1 to R3 were put into three chambers with 50 mL of the synthetic wastewater as given above. Each chamber has a working volume of 80 mL. Before the SOUR test, biomass on GAC was measured in order to have the same initial biomass concentrations of 1 g L^{-1} in the chambers. The temperature was maintained at 25°C.

In order to determine the main elemental compositions of the biofilms developed on GAC, biofilm samples were dried to a constant weight by the freeze dryer (Martin Christ, BETA 1-8 LDplus, Germany). 1.0 mg of the dried biofilm was used to determine the C, H, N and O contents by the CHNS analyzer (PerkinElmer, 2400 II, USA). The AMP concentration in solution was determined by HPLC (Perkin Elmer

Series 200, USA) as described in Chapter 3. Concentrations of total organic carbon (TOC) in the influent and effluent were analyzed by TOC analyzer (Shimadzu, TOC-Vcsh, USA).

4.3 RESULTS AND DISCUSSION

4.3.1 Formation of Biofilm on GAC

Figure 4.2 shows the changes in the biofilm dry weight on unit GAC in three reactors. It was found that the trends of the biofilm growth in three reactors were subject to a similar pattern, i.e. the initial phase before day 14, the growing phase from day 14 to 30, followed by the stable phase from day 30 onwards. After 30 days of operation, the biofilms developed in R1 to R3 stabilized at 40 to 50 mg g^{-1} GAC. It appears from Figure 4.2 that AMP in the concentration range studied would not have negative effect on the biofilm development on GAC. On the contrary, AMP, to some extent, would serve as a promoter to stimulate the biofilm growth.

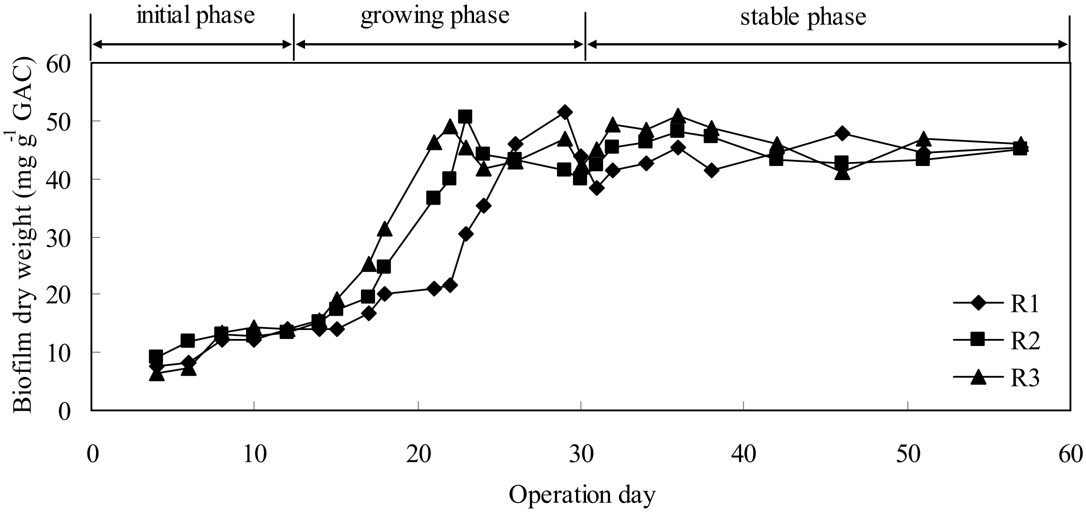
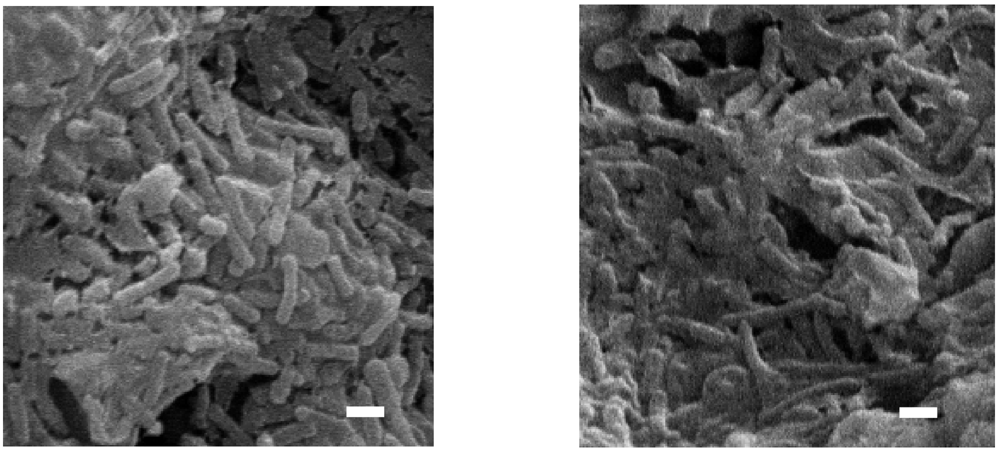


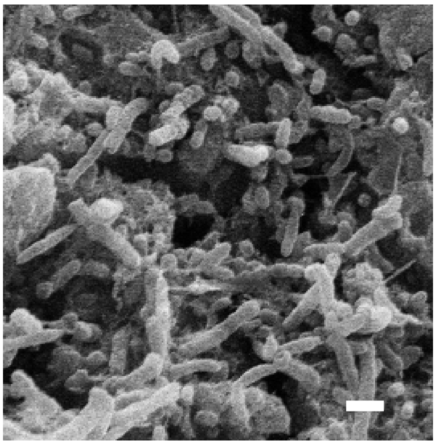
Figure 4.2 Development of biofilms in R1 to R3.

SEM images of the mature biofilms taken from R1 to R3 show different types of bacteria, such as rod, cocci and filamentous bacteria embedded in the EPS matrix on the surface of the GAC (Figure 4.3).



(a) R1: AMP 0 mg L^{-1}

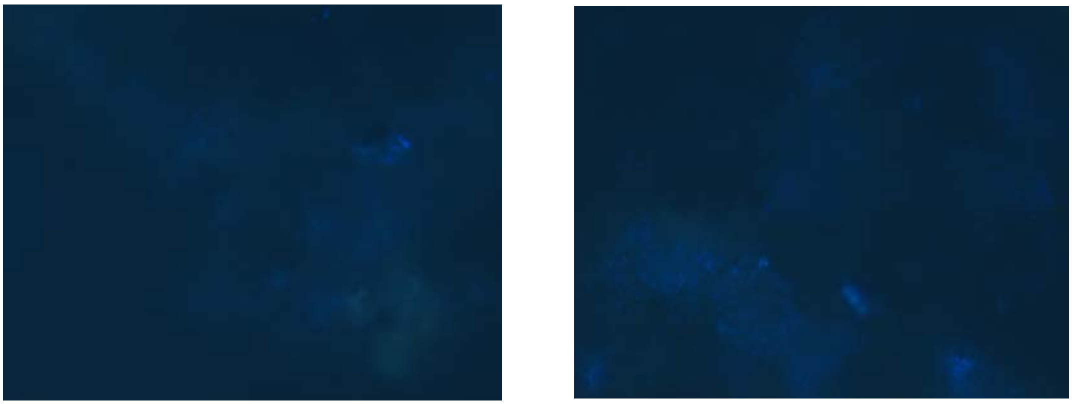
(b) R2: AMP 4 mg L^{-1}



(c) R3: AMP 8 mg L^{-1}

Figure 4.3 Morphology of mature biofilms in R1 to R3 on day 38 (Bar: 1μm).

DAPI staining was performed on the biofilms developed on GAC with 8 mg L^{-1} AMP on different operation days. The coverage of the immobilized bacteria on GAC was visualized by epifluorescence microscopy from day 4 onwards (Figure 4.4). It was found that biofilms extensively developed on the GAC surface after 38 days of operation (Figure 4.4c).

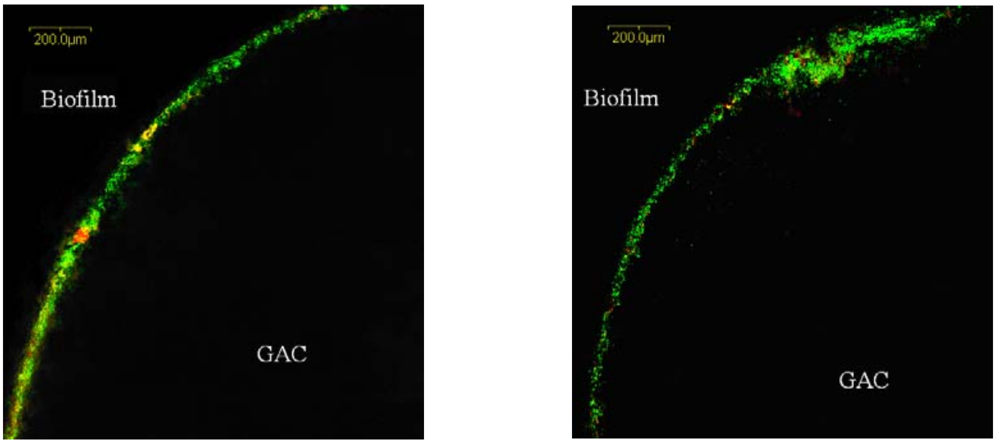


(a) day 4 (b) day 10

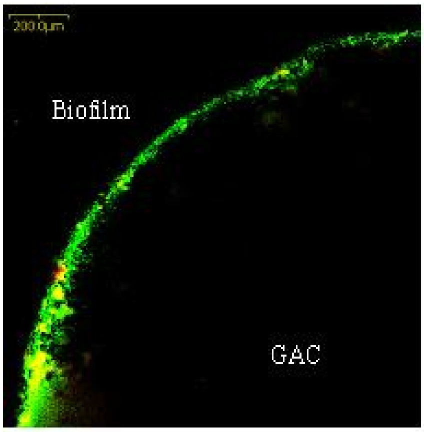
(c) day 38

Figure 4.4 Epifluorescence microscopy images of immobilized bacteria on GAC in R3 fed with 8 mg L^{-1} AMP. DAPI-staining; 100 × magnification.

Figure 4.5 further shows the CLSM images of the mature biofilms on the sectioned BGAC from R1 to R3, in which live and dead cells were differentiated by green and red florescence, respectively. It was found that live microorganisms mainly situated at the surface of GAC, and very few dead cells were detected in the biofilms developed with or without exposure to AMP. These in turn suggest that these biofilms on GAC would be highly viable. Since the biofilm was not evenly distributed on GAC, and its accurate measurement was therein impossible. However, as shown in Figure 4.5, the biofilm thickness should be in the range of 50 to 200 μm.



(a) R1: AMP 0 mg L^{-1} (b) R2: AMP 4 mg L^{-1}



(c) R3: AMP 8 mg L^{-1}

Figure 4.5 CLSM images of biofilms developed in the presence and absence of AMP on day 38. Green: live cells; red: dead cells; dark: none of cells.

EPS in Biofilm

So far, it has been well known that extracellular polymeric substances (EPS) are the important components to maintain the structural integrity, and facilitate cell-to-cell interaction in biofilms (Flemming and Wingender 2001; Sutherland 2001). Figure 4.6 shows changes in the ratio of extracellular polysaccharide (PS) to extracellular protein (PN) in the course of the reactor operation. It can be seen that the PS/PN ratio increased sharply when the biofilms grew from the initial phase into the growing phase in R1 to R3, respectively. In contrast, a declined PS/PN ratio was observed along with the maturation of the biofilms, and gradually stabilized at 0.4

mg/mg in R1 to R3. A similar phenomenon was also observed in the *Pseudomonas fluorescens* biofilm formed on basalt in an airlift reactor (Lopes et al. 2000), as well as in a granular sludge biofilm SBR (Jiang and Liu 2010). These seem to imply that extracellular polysaccharides would be more important for initial microbial attachment onto a solid surface, whereas extracellular proteins may contribute more to the bacterial interaction or aggregation in the mature biofilms.

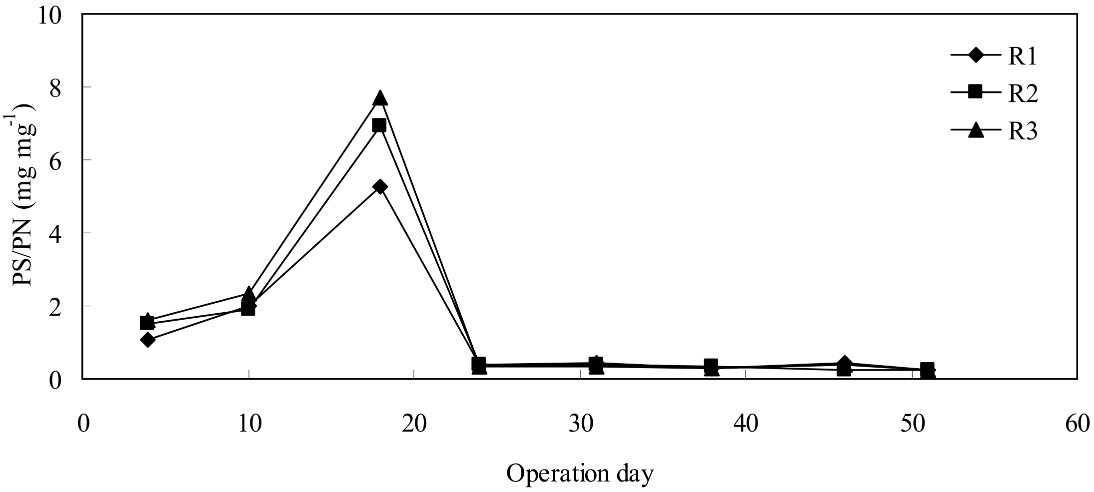


Figure 4.6 PS/PN ratio versus operation time.

In the study of the influence of cell surface properties of *thermophilic streptococci*, Flint et al. (1997) found that disrupting cell surface polysaccharides with sodium metaperiodate, lysozyme, or trichloroacetic acid had no effect on its attachment to stainless steel, whereas removing cell surface proteins by trypsin or sodium dodecyl sulfate resulted in a 100-fold reduction in the number of attaching bacteria. Dufrene et al. (1996) investigated the adhesion of *Azospirillum brasilense* to glass, and observed a correlation between the protein concentration and adhesion density under different experimental conditions, indicating the involvement of extracellular protein in the attachment of *A. brasilense* to inert surface. Recently, Jiang et al. (2010) also found that the PN content was 3-fold higher than PS in the mature aerobic granular sludge biofilm, and thought that the disintegration of the granular sludge biofilm was related to reduced PN content. It is apparent that specific contribution of PN and PS to microbial attachment is subject to further study. It should be pointed out that the role of PS, to some extent, is over highlighted against PN in biofilm formation.

The peak PS/PN values for the biofilms developed in R1 to R3 seem to be AMP concentration-dependent, i.e. a higher PS/PN value was observed at a higher AMP concentration. This observation can be ascribed to the response of microbes to AMP when forming biofilms. Arciola et al. (2005) investigated the prevalence of 342 clinical isolates of *Staphylococcus epidermidis* on the agar plates with various kinds of antibiotics, including 126 PS-producing strains and 216 non PS-producing strains, and found a pronounced difference between these two groups of bacteria, i.e. the PS-producing strains showed greater prevalence for all the tested antibiotics. Therefore, it is reasonable to consider that secretion of PS by microorganisms subject to the stress of AMP would be an effective defensive strategy against the AMP toxic or inhibitory effect on the biofilm growth.

Microbial Activity of Biofilm

In aerobic processes, molecular oxygen serves as the final electron acceptor. Therefore the metabolic activity of a microbial community is directly related to the respiratory activity of cells. In this study, the respirometer was used to determine the specific oxygen utilization rate (SOUR) of the biofilms developed in R1 to R3 (Figures 4.7 and 4.8). For this purpose, the BGAC samples were collected on day 10 and 42 from R1 to R3.

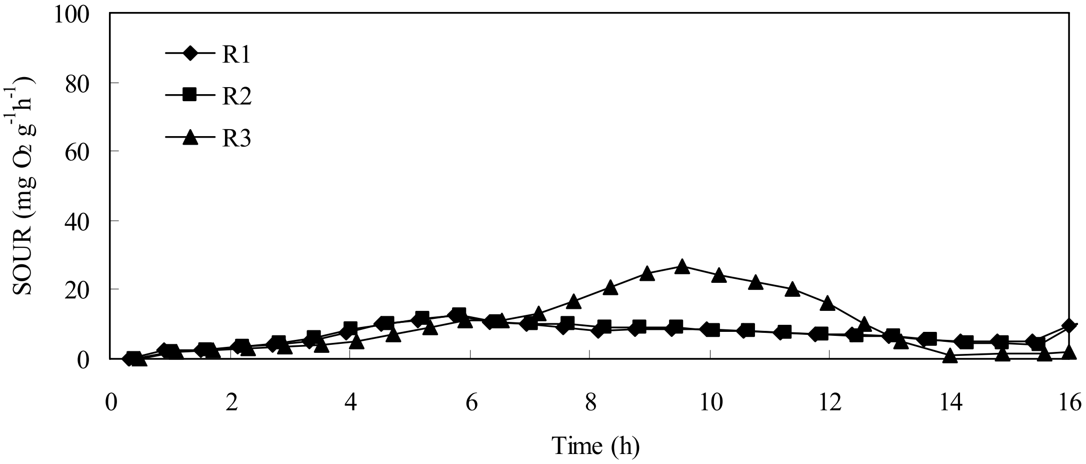


Figure 4.7 SOUR profiles of the biofilms developed on GAC on day 10.

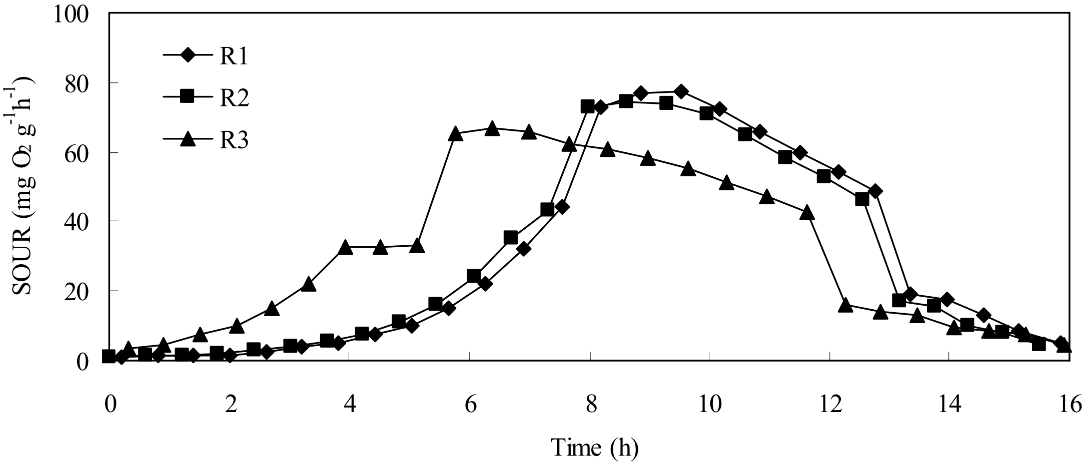


Figure 4.8 SOUR profiles of the biofilms developed on GAC on day 42.

The SOUR values of the biofilms on day 10 were much lower than those on day 42 for all the three reactors. It appears from Figures 4.7 and 4.8 that that total oxygen consumption rate was increased by 4.4, 4.2 and 3.2 folds for R1, R2 and R3 from day 10 to 42, respectively. In fact, such observation is in line with increased biofilm dry weight on GAC from day 10 to day 42 (Figure 4.2). With more biofilms accumulated on GAC, there should be more exposed surface of bacteria to utilize oxygen. Concerning the effect of AMP on the respiratory activity of biofilms, it is seen that SOUR profiles in three reactors were almost overlapped (Figures 4.7 and

4.8), i.e., the respiratory activity of the biofilm developed with AMP was almost the same as that without AMP. It appears that AMP did not inhibit the bacterial activity in this study, consisting with the live cells distribution in Figure 4.5. In a previous study about the anaerobic treatment of antibiotic erythromycin, Amin et al. (2006) also found that erythromycin at the tested concentration would not affect the microbial activity in terms of the biogas production. It has generally been perceived that the effect of antibiotics on microorganisms is concentration-dependent, which can be characterized by a minimum inhibitory concentration (MIC), i.e. the lowest inhibitory concentration for microbial growth (Andrews 2001). The MIC of AMP has been reported to be in the range of 8 to 16 mg L^{-1} for most bacteria (Lorian 1996; Butler et al. 1999). As the highest AMP concentration used in this study was 8 mg L^{-1}, it is natural to observe that AMP at such concentration would not inhibit the biofilm growth as compared to the control reactor free of AMP (R1) (Figure 4.2). This is also evidenced by comparable SOUR values as shown in Figures 4.7 and 4.8 as well as the microscopic observation (Figures 4.5).

Instead of inhibitory effect, AMP appears to play a trigger role for the biofilm development (Figure 4.2) as well as PS secretion in the growing phase of the biofilm (Figure 4.6). It appears from the study of medical microbiology that antibiotics in the subinhibitory level (i.e. below MIC) can induce biofilm formation by stimulating the production of EPS (Rachid et al. 2000; Bagge et al. 2004; Majtán et al. 2008), or by increasing bacterial growth (Lorian 1993; Hoffman et al. 2005). Moreover, it has been reported that antibiotics can serve as alternative signaling molecules involved in quorum sensing which is one of the main mechanisms for biofilm development (De Kievit 2009). As noted by Linares et al. (2006), antibiotics may be more than weapons for destroying competing microbes, and they may also act as signaling molecules regulating bacterial gene expression and enabling microbial communities to coordinate an adaptive response to an ever-changing environment.

Shen et al. (2008) also examined the response of strain *Pseudomonas aeruginosa* PAO1 to the subinhibitory AMP in term of the regulation virulence gene, and found that the expression of gene *rhlAB* was up-regulated 7.7-fold in the presence of 1.95 mg L^{-1} AMP, meanwhile the bacterial growth in the assay plate was not affected by the subinhibitory AMP (Figure 4.9). In fact, previous microbiological study showed that gene *rhlAB* was involved in the biofilm formation (Davey et al. 2003). These seem to imply that AMP at its subinhibitory concentration would induce biofilm formation by signaling mechanism. Moreover, Shen et al. (2008) found that the production of extracellular molecules, such as rhamnolipids and pyocyanin in the presence of 1.95 mg L^{-1} AMP was also increased by 77% and 78% respectively, compared to that in the absence of AMP. To date, the effect of subinhibitory antibiotics on the microorganism activation is not fully understood.

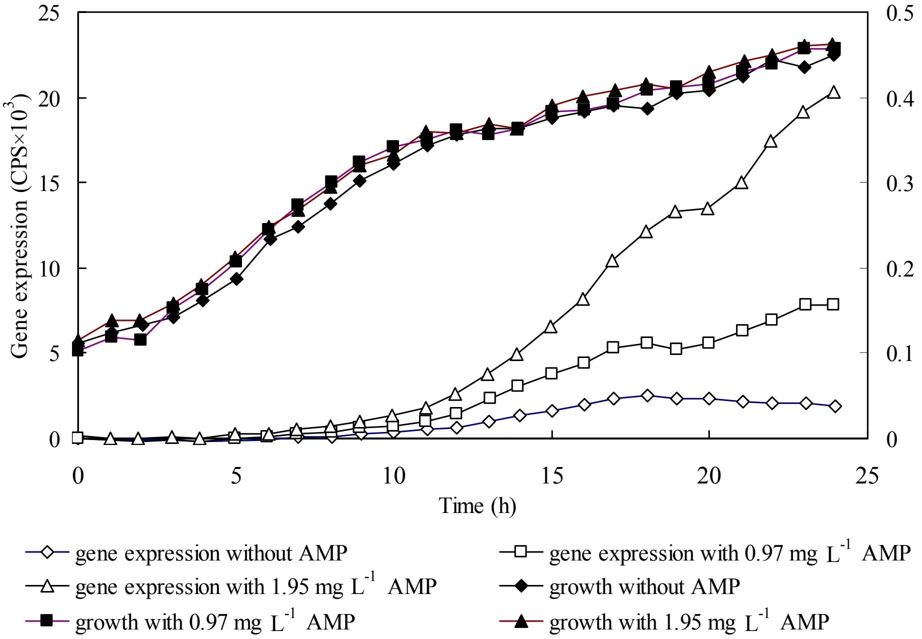


Figure 4.9 Gene expression profiles and growth of *Pseudomonas aeruginosa* PAO1 with reporter pKD-*rhlAB* in the presence or absence of subinhibitory AMP. Data from Shen et al. (2008).

Chemical Composition of Biofilm

The elemental composition of microorganisms reflects the energy and nutrients flow within the microorganisms under given culture conditions. After R1 to R3 reached the steady state, the respective contents of carbon (C), hydrogen (H), nitrogen (N), sulphur (S) and oxygen (O) in the biofilms developed in R1 to R3 were analyzed (Table 4.2). It can be seen that the cellular C/N ratio decreased from 4.6 to 4.2 with the increase in the AMP concentration from 0 to 8 mg L^{-1} in R1 to R3.

Table 4.2 Elemental composition of biofilms in % by dry weight.

Element	R1	R2	R3
C	40.01	38.2	33.85
H	7.23	6.99	4.67
N	8.76	8.71	8.05
S	2.27	1.7	2.82
C/N	4.6	4.4	4.2

The growth conditions and differences in the bacteria community can cause the variability of elemental composition (Reeslev et al. 1996; Vrede et al. 2002). As noted by Elser et al. (2000), the variation in cell elemental compositions would imply how species-interactions develop in ecosystems under various conditions of energy input and nutrient supply. It appears that AMP would have the significant influence on the microbial composition and distribution in the biofilm ecosystem, which may lead to the distinct biodegradation capabilities, as further demonstrated in Chapter 5.

4.3.2 Reactor Performance

Figure 4.10 shows the respective concentration profiles of TOC and AMP observed in R1 to R3. Three salient points appear from Figure 4.10: (i) the TOC removal in R1 to R3 was subject to a similar pattern regardless of the addition of AMP; (ii) 90% of TOC removal was achieved in all the three reactors, and (iii) AMP in the effluent was undetectable, i.e. below the detection limit of HPLC used in this study (10 μg L^{-1}).

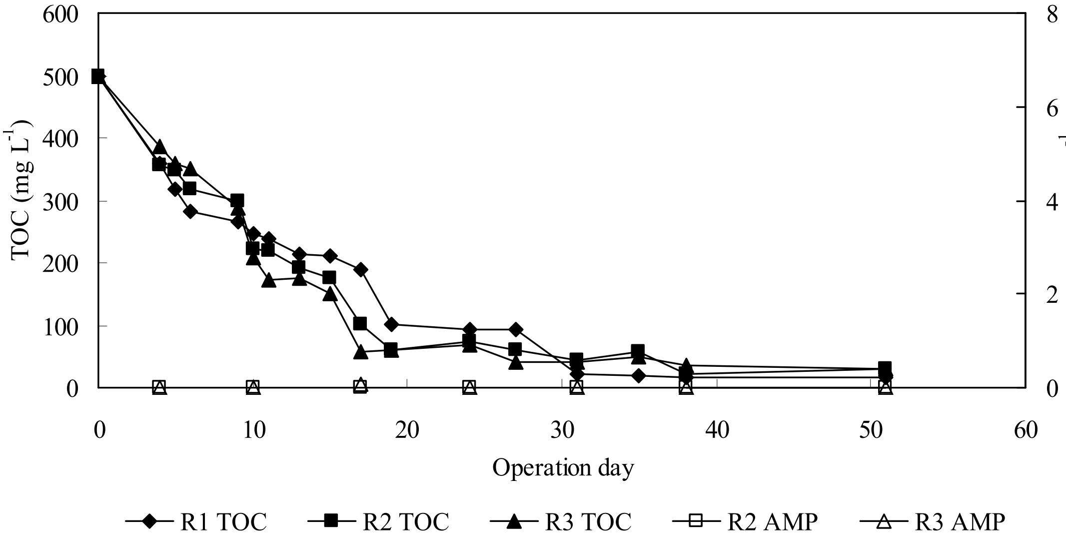


Figure 4.10 Concentration profiles of TOC and AMP in R1 to R3.

In fact, β-lactam antibiotics are not readily degradable by suspended microorganisms (Al-Ahmad et al. 1999; Alexy et al. 2004). Moreover, in study of biodegradation of AMP in a biofilm airlift reactor using ceramic beads as carrier, Zhou et al (2006) observed less than 10% of AMP removal at various HRTs. Obviously, compared to ceramic beads, GAC has the high adsorption capacity as shown in Chapter 3. This indicates that GAC would significantly contribute to the observed AMP removal through adsorption in this study, especially at the initial stage of the biofilm development.

4.3.3 Mechanisms of AMP Removal by BGAC

In order to evaluate the possible mechanisms of AMP removal in the continuous bioreactor, the batch experiments were conducted with the BGAC collected from R2 and R3 on different operation days. Figures 4.11 and 4.12 show the AMP concentration profiles observed in the batch experiments with the initial AMP concentrations of 4 and 8 mg L^{-1}, respectively. In general, AMP can be removed by BGAC through three possible mechanisms: adsorption by GAC, adsorption by biofilms and biodegradation by biofilms. It appears from Figures 4.11 and 4.12 that the observed AMP removal can be reasonably described by a first-order kinetics, i.e.,

$$r = -\frac{dC}{dt} = r_{ad\text{-}GAC} + r_{ad\text{-}biofilm} + r_{bio} = k_1 C \tag{4.1}$$

in which r is the overall removal rate of AMP; C is AMP concentration in solution; $r_{ad\text{-}GAC}$, $r_{ad\text{-}biofilm}$ and r_{bio} are the rates for adsorption by GAC, adsorption by biofilms and biological removal of AMP, respectively; and k_1 is the overall rate constant.

To examine the possible AMP adsorption by biofilms, biofilms collected from 5 grams of mature BGAC were autoclaved to deactivate the biological activity. The treated biomass was used in the batch adsorption experiment with an initial AMP concentration of 8 mg L^{-1}. It can be seen in Figure 4.13 that the AMP adsorption by biofilms was much slower than those observed in Figures 4.11 and 4.12. This suggests that the term $r_{ad\text{-}biofilm}$ in Eq. 4.1 can be ruled out. Thus, Eq. 4.1 reduces to

$$r \approx r_{ad\text{-}GAC} + r_{bio} = k_1 C \tag{4.2}$$

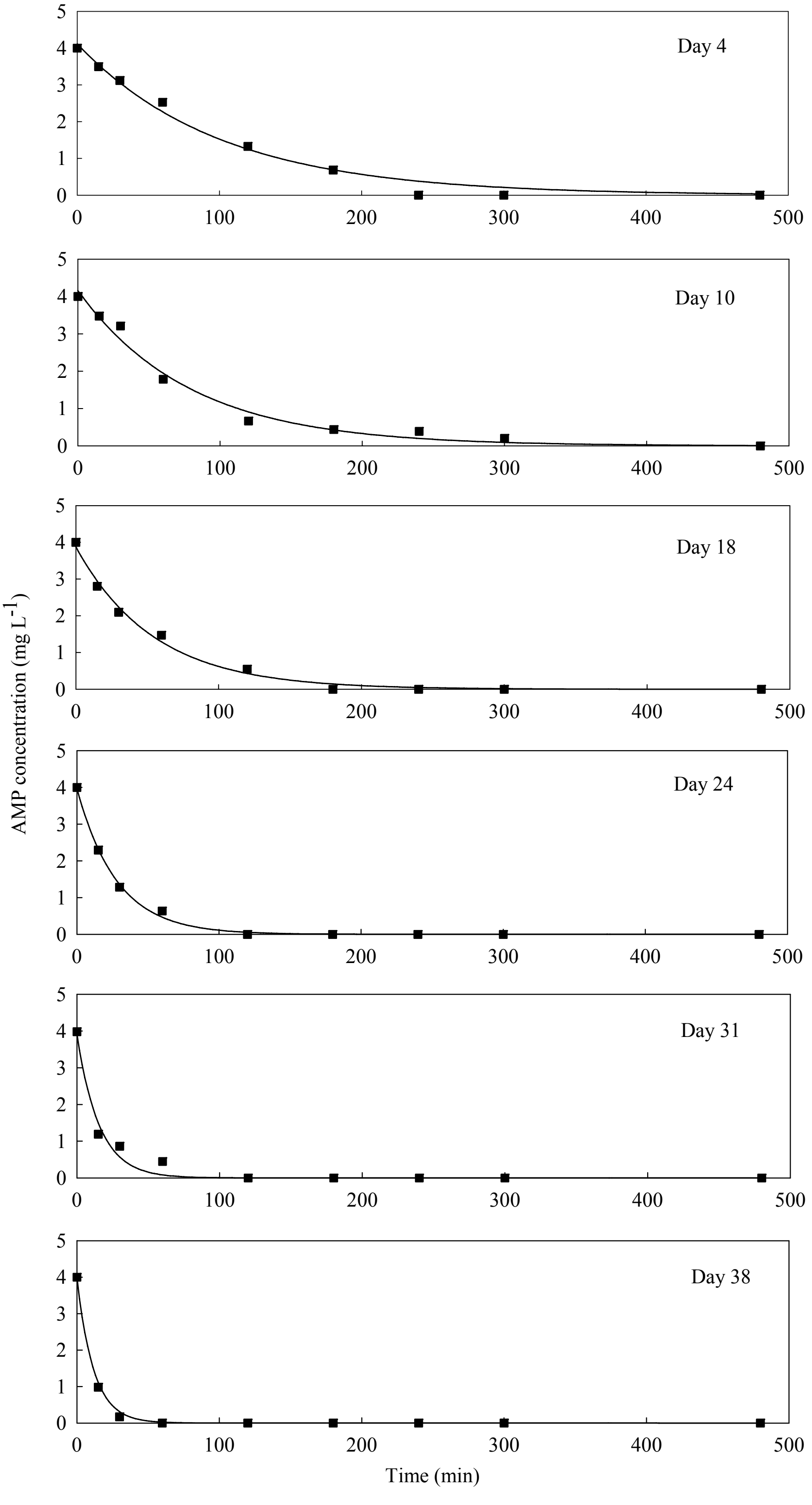


Figure 4.11 AMP concentration profiles in the batch experiments with BGAC taken from R2 on different operation days, C_0=4 mg L^{-1}. ■: experimental data; solid line: prediction by Eq. 4.6.

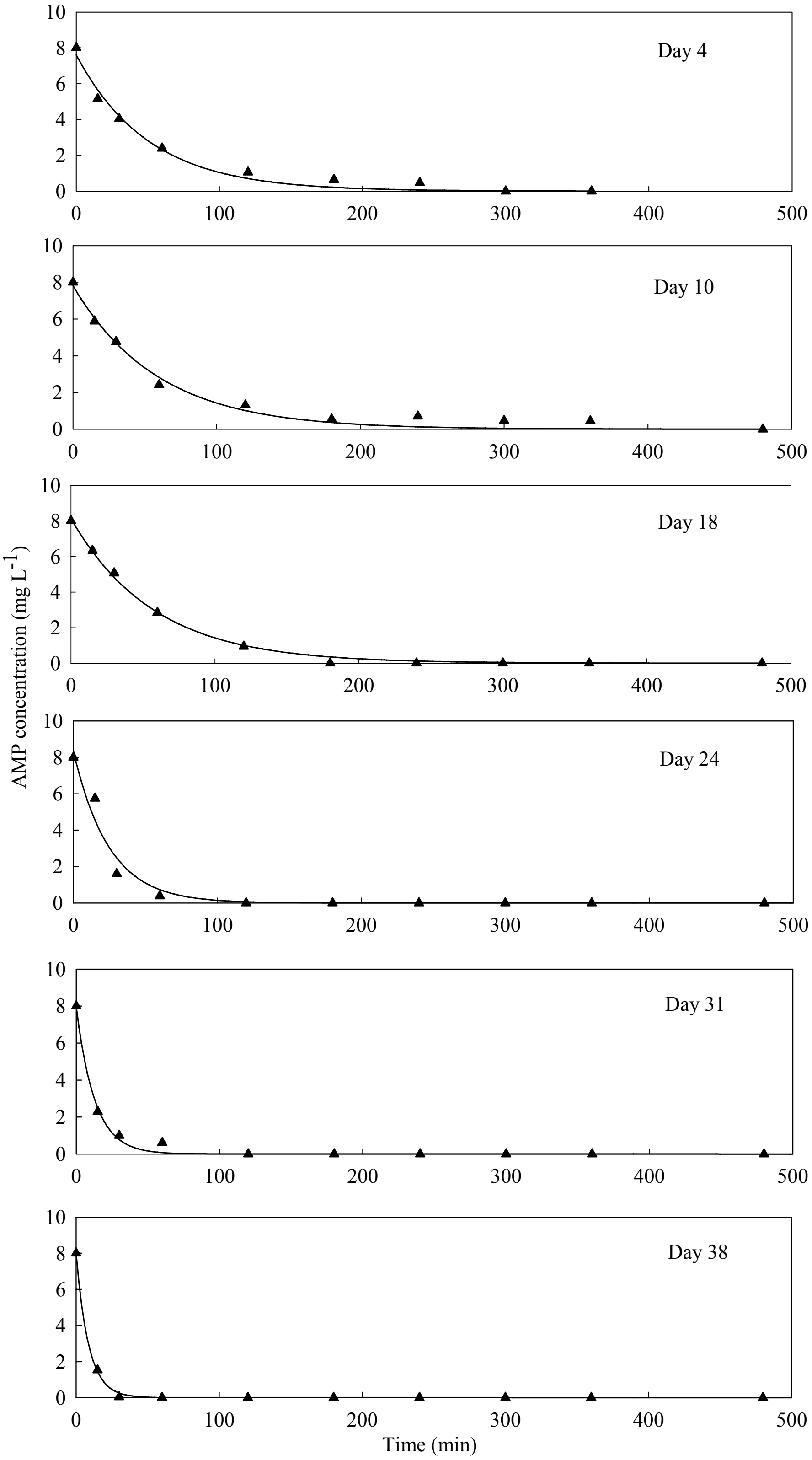


Figure 4.12 AMP concentration profiles in the batch experiments with BGAC taken from R3 on different operation days, C_0=8 mg L^{-1}. ▲: experimental data; solid line: prediction by Eq. 4.6.

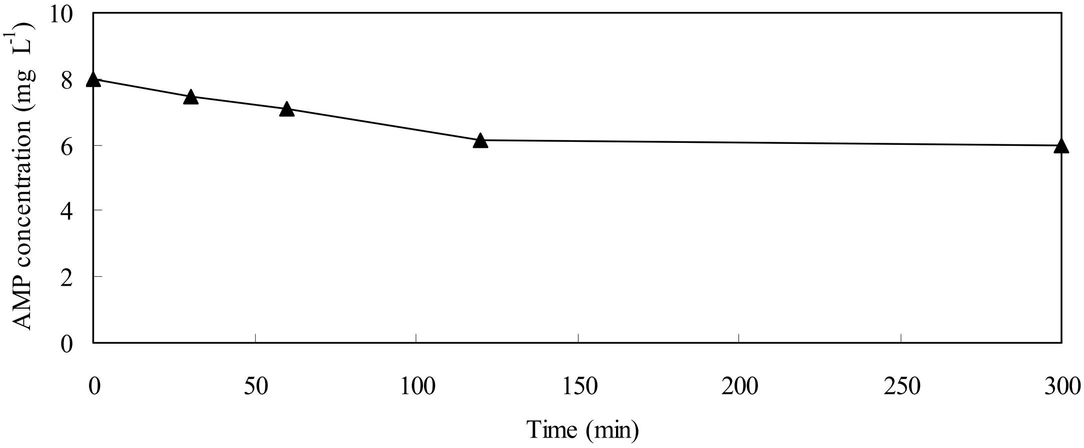


Figure 4.13 AMP adsorption by the biofilms detached from the R3 BGAC on day 38.

As discussed in Chapter 3, the AMP adsorption by GAC at the initial concentrations lower than 518 mg L^{-1} (Table 3.6) follows the first-order rate equation:

$$r_{\text{ad-GAC}} = k_{\text{ad-GAC}} C \tag{4.3}$$

in which $k_{\text{ad-GAC}}$ is the rate constant of GAC adsorption. Figure 4.2 showed no AMP inhibition to the biofilm development, hence the Monod equation is applicable for the description of the biological removal of AMP by BGAC, i.e.

$$r_{\text{bio}} = r_{\text{bio,max}} \frac{C}{C + K_C} \tag{4.4}$$

in which $r_{\text{bio,max}}$ and K_C are maximum biological removal rate and a constant, respectively.

According to Alexander (1999), C is often smaller than K_C in the biological treatment of recalcitrant organic compounds, i.e. Eq. 4.4 can be approximated by

$$r_{\text{bio}} \approx k_{\text{bio}} C \tag{4.5}$$

in which $k_{\text{bio}}=r_{\text{bio,max}}/K_C$. Equation 4.5 represents a first-order rate equation for the biological removal of AMP. Anderl et al. (2000) also reported that biodegradation of AMP by planktonic cultures of *Klebsiella pneumoniae* was subject to the first-order kinetics even at the AMP concentration as high as 1000 mg L^{-1}. In addition, Eq. 4.5 has been applied widely to describe the biodegradation kinetics of various antibiotics (Fitch et al. 1996; Ingerslev et al. 2001; Degirmentas and Deveci 2004).

Substitution of Eqs. 4.3 and 4.5 into Eq. 4.2 gives:

$$r = (k_{ad\text{-}GAC} + k_{bio})C = k_1 C \quad (4.6)$$

in which

$$k_1 = k_{ad\text{-}GAC} + k_{bio} \quad (4.7)$$

Figures 4.11 and 4.12 show that Eq. 4.6 can provide an excellent description for all the experimental data obtained, indicated by the correlation coefficients higher than 0.95.

The k_1 values determined on different operation days are presented in Figures 4.14. and 4.15. It is reasonable to consider that with the development of biofilms on GAC, the adsorption of AMP by such GAC would slow down due to the additional diffusion barrier of biofilms on GAC. As the result, $k_{ad\text{-}GAC}$ would be expected to be reduced along with the biofilm development on GAC. These in turn indicate that the observed increase in k_1 (Figures 4.14 and 4.15) would be attributed to the increased k_{bio} over the operation day, as reflected in Eq. 4.7, i.e. biodegradation would significantly contribute to the observed AMP removal for the mature BGAC. The effect of the biomass development on k_1 was further shown in Figures 4.16 and 4.17. It can be seen that k_1 tends to increase with the increase in the amount of biofilms. This further confirms that biodegradation capability of biofilms to AMP was built-up along with the biofilm development on GAC since biosorption of AMP was negligible (Figure 4.13).

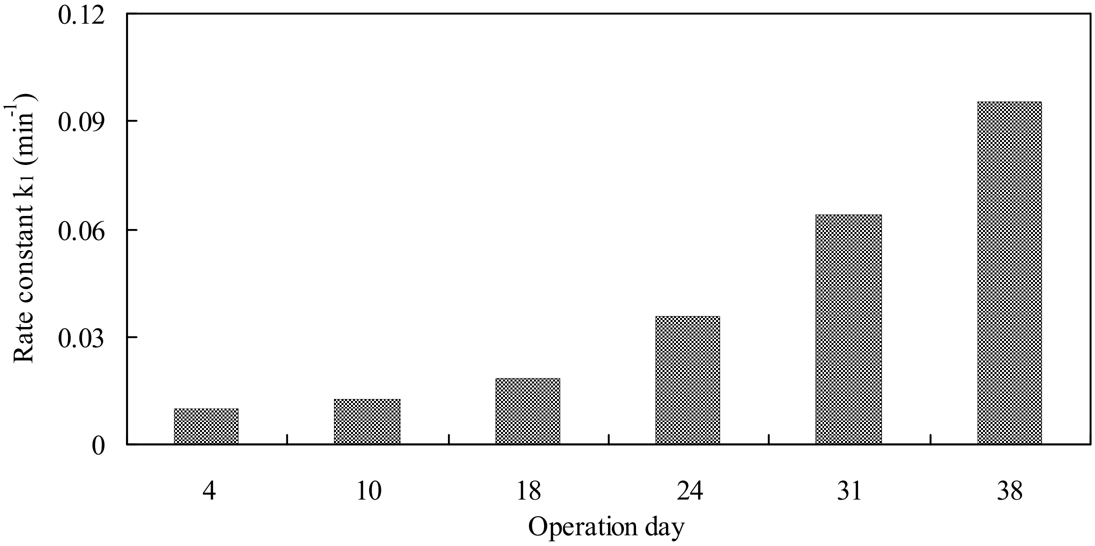


Figure 4.14 Rate constants of AMP removal by BGAC taken from R2 fed with 4 mg L^{-1} AMP.

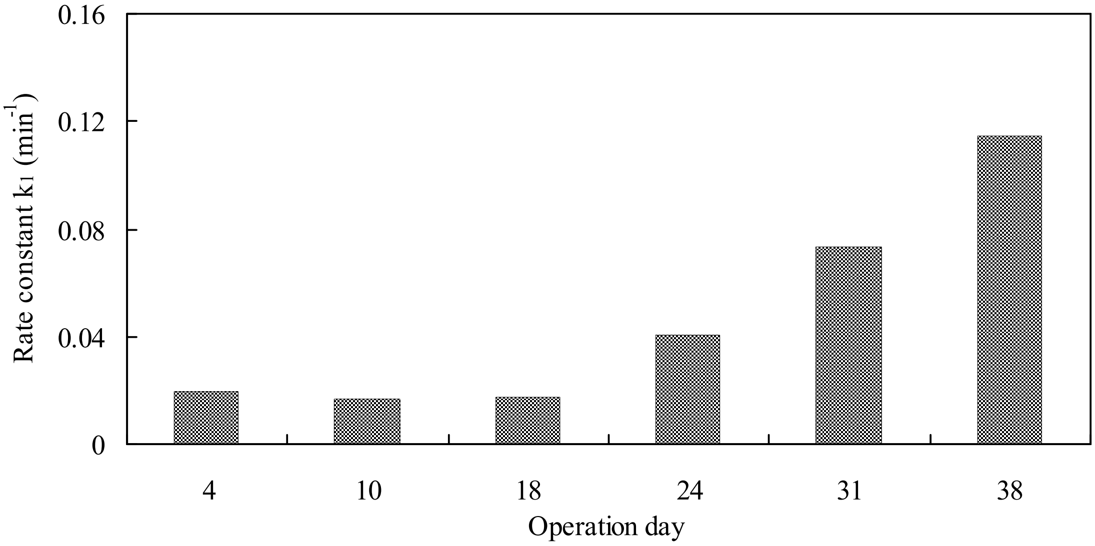


Figure 4.15 Rate constants of AMP removal by BGAC taken from R3 fed with 8 mg L^{-1} AMP.

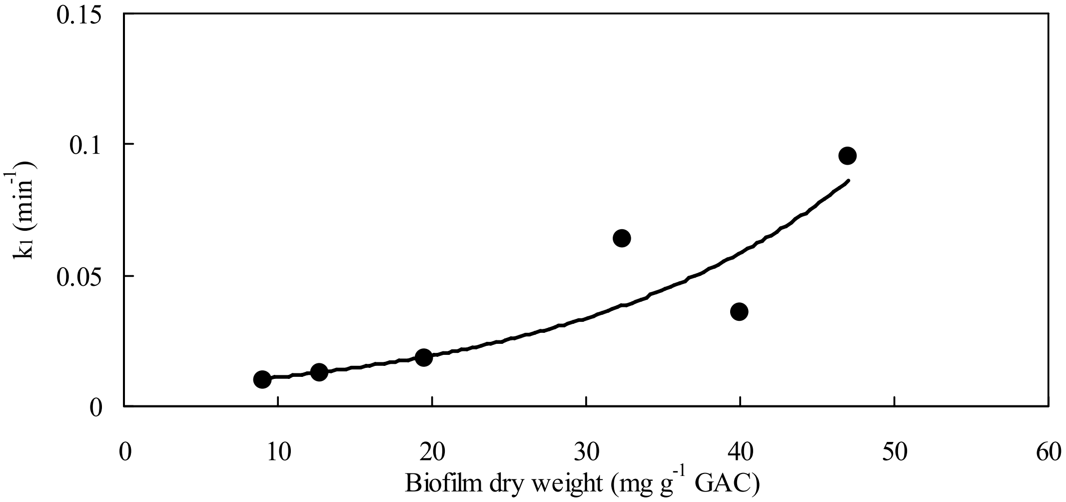


Figure 4.16 Correlation of k_1 and the biofilm dry weight for BGAC taken from R2 fed with 4 mg L^{-1} AMP.

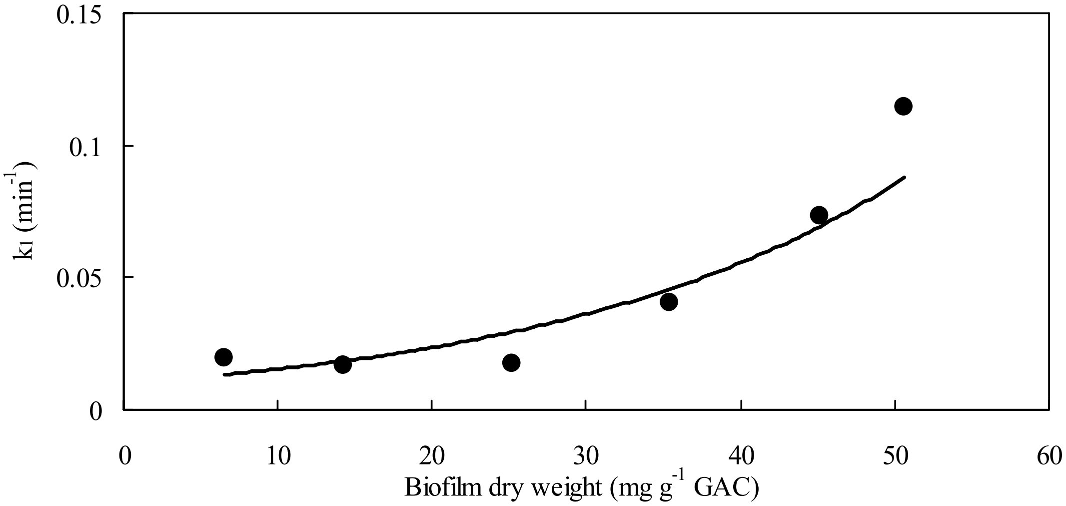


Figure 4.17 Correlation of k_1 and the biofilm dry weight for BGAC taken from R3 fed with 8 mg L^{-1} AMP.

As the biofilm activity in the initial phase is very low (Figure 4.7), it would be reasonable to consider that $k_{ad\text{-}GAC}$ should be close to k_1 determined on day 4. Therefore, for the mature BGAC, the biodegradation potential of AMP would be much higher than that of adsorption by GAC. Drillia et al. (2005) reported that sulfamethoxazole, a common antibiotic, was biologically removed by activated sludge under aerobic conditions, whereas 35% of ibuprofen was degraded by an oxic biofilm (Zwiener and Frimmel 2003). Ingerslev et al. (2001) also found that the primary biodegradation of antibiotics olaquindox (OLA), metronidazole (MET), tylosin (TYL) and oxytetracycline (OTC) in an antibiotics-polluted surface water followed the first-order kinetics, and the half-lives for aerobic biodegradation of the antibiotics ranged from 4 to 8 days for OLA, 9.5 to 40 days for TYL, 14 to 104 days for MET and 42 to 46 days for OTC. The long half-life would be due to extremely low bacteria concentration present in the surface water. For example, addition of 3 mg L^{-1} of activated sludge from wastewater treatment plant would enhance the biodegradation potential which was believed to be the result of increased bacterial concentration in the shake flask tests, whereas biodegradation of the antibiotics was significantly slow in tests conducted in absence of oxygen (Ingerslev et al. 2001).

For a pure GAC adsorber, i.e. without biofilm growth, AMP can only be removed through adsorption. In this case, the AMP concentration at the adsorption equilibrium can be predicted through the operation line method (Ho and McKay 1998). As shown in Figure 4.18, the initial and final coordinates of the operation line are (C_0, q_0=0) and (C_e, q_e) and the slope is the reciprocal of the adsorbent dose, i.e. 1/X. Combination of the operation line with the Langmuir isotherm allows to calculate the expected adsorption capacity ($q_{e,predict}$):

$$\begin{cases} \text{operation line: } q_{e,predict} = \dfrac{C_0 - C_{e,predict}}{X} \\ \text{Langmuir isotherm: } q_{e,predict} = q_m \dfrac{K_L C_{e,predict}}{K_L C_{e,predict} + 1} \end{cases} \tag{4.8}$$

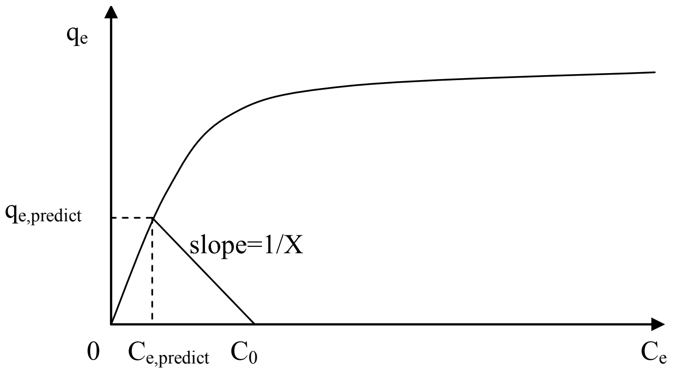


Figure 4.18 Adsorption isotherm and the operation line for a given adsorbent dose (X).

As shown in Chapter 3, Langmuir isotherm is applicable for the AMP adsorption by GAC. Therefore, the theoretical adsorption capacity of AMP by total GAC dosed in the reactor ($W_{ad,AMP}$) can be calculated as

$$W_{ad,AMP} = q_{e,predict} M_{GAC} \tag{4.9}$$

in which M_{GAC} is the total amount of GAC in the reactor. The total amount of the input AMP ($W_{in,AMP}$) to the reactor can be determined as follows:

$$W_{in,AMP} = C_0 Qt \tag{4.10}$$

in which Q is the influent flow rate, and t is the reactor operation time. For C_0=4 mg L^{-1} and C_0=8 mg L^{-1}, $W_{ad,AMP}$ was compared with $W_{in,AMP}$ over the experimental period (Table 4.3). It appears that the amount of the input AMP to the reactor was nearly 1.8-fold higher than the amount of AMP that could be adsorbed by GAC. Therefore, if the adsorption of AMP by GAC is the sole mechanism for the observed AMP removal in the biofilm reactor, all the GAC in the reactor would be saturated when $W_{in,AMP}$=$W_{ad,AMP}$, i.e. from day 34 onwards as can be seen in Figure 4.19. Moreover, under the assumption of pure adsorption of AMP by GAC, the effluent AMP concentration would be much higher than the actual measured concentration (Figure 4.10). These clearly indicate that GAC adsorption alone can not provide a plausible explanation for the undetectable AMP concentration in the effluent from the biofilm reactor, instead suggest a strong role of AMP biodegradation by BGAC in the overall AMP removal.

Table 4.3 Comparison of the maximum adsorption capacity of AMP by GAC and the total amount of AMP fed to R2 and R3 over 60 days of operation.

C_0 (mg L^{-1})	$q_{e,predict}$ (mg g^{-1})	$W_{ad,AMP}$(mg)	$W_{in,AMP}$ (mg)	$W_{in,AMP}/W_{ad,AMP}$
4	6.11	1375	2400	1.75
8	12.02	2704	4800	1.78

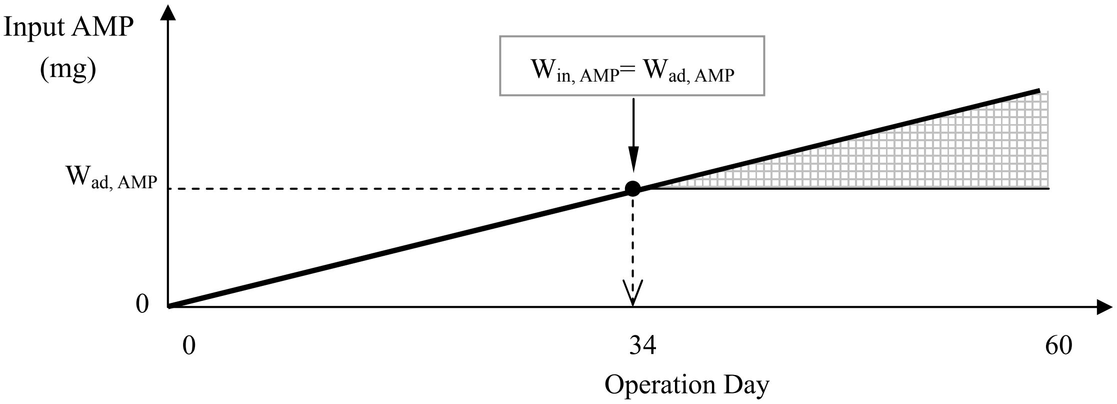


Figure 4.19 Prediction of the exhaustion of a pure GAC adsorption.

The full adsorption capacity of pure GAC would be exhausted when the adsorption equilibrium is reached. However, for BGAC, the adsorbed AMP can be further removed through biodegradation by the biofilms developed on GAC; such a process is known as bio-regeneration. Bio-regeneration is often defined as the renewal of the adsorptive capacity of activated carbon by microorganisms for further adsorption (Aktas and Cecen 2007). In fact, there are another two well-established methods for the regeneration of exhausted carbon except for bio-regeneration, i.e. thermal and chemical regeneration. Thermal regeneration, the most widely used regeneration technology, has an important energy demand since temperature must be kept above 1100 K, as well as the special instruments, such as rotary kiln and multiple hearth furnaces, are needed (San Miguel et al. 2001). Chemical regeneration can be carried out by using specific solvents, requiring further extraction and purification, whereas the solvent recovery would be very difficult when ketones are present (Thomson and Crittenden 1998). Compared to the conventional thermal and chemical regeneration methods, bio-regeneration is more devise-effective and cost-effective, and thus arises as an appealing regeneration technology for exhausted GAC. So far, two hypotheses have been proposed to explain bio-regeneration of the exhausted GAC. One hypothesis is that the adsorbed organics are released from the carbon due to the concentration gradient, and subsequently degraded by microbial activities (Speitel Jr et al. 1989; de Jonge et al. 1996). The second hypothesis is that exoenzymes excreted by microorganisms may diffuse into the pores of activated carbon, and then react with adsorbed substances (Perrotti and Rodman 1974; Kim et al. 1997). Although opinions on these two hypotheses are still debatable, it is widely accepted that biodegradation of the adsorbed substance is the key step in a successful bio-regeneration.

The results obtained in this study show that bio-regeneration can occur in a simultaneous AMP adsorption-biodegradation cycle as the reactor is running, in which the bio-regeneration efficiency should be especially dependent on the biodegradation potential other than the equilibrium adsorption capacity for the mature BGAC. It is obvious that AMP biodegradation is the leading mechanism towards the bio-regeneration observed in the BGAC bioreactor, i.e. the sustainable bio-regeneration of BGAC is feasible for the long-term operation. Moreover, the porous GAC with high adsorptive capacity (Chapter 3) can act as a sink or buffer, meaning that the AMP adsorbed by GAC in the initial phase of the biofilm development can be later biodegraded by the microorganisms grown on GAC. Therefore, the BGAC developed in the airlift bioreactor has the capability of bio-regeneration, leading to a sustainable uptake of AMP by BGAC. This is the beauty of the BGAC system developed in this study.

4.4 SUMMARY

Biofilms were developed on GAC in the continuous airlift reactors supplemented with AMP of 4 and 8 mg L^{-1}, respectively. Over 60 days of operation, AMP was not detected in the effluents from R2 and R3. Compared to the control reactor, AMP at the concentration levels studied did not exhibit influence on the biofilm development, biofilm morphology and viability. However, AMP would stimulate the biofilm growth and the secretion of extracellular polysaccharide in the growing phase; meanwhile it also altered the elemental composition of the mature biofilms. These findings suggest that AMP may exert a selection pressure on microbial species in the biofilms developed on GAC. It was revealed that the observed AMP removal was mainly attributed to the adsorption by GAC and biodegradation by the biofilms. However, the biodegradation capability of the biofilms was gradually built up in the course of the reactor operation. It was also demonstrated that the BGAC developed in the airlift bioreactor underwent the simultaneous bio-regeneration which allows a sustainable operation of the system.

CHAPTER 5

BACTERIAL COMMUNITY OF THE BIOFILMS DEVELOPED WITH AMPICILLIN

5.1 INTRODUCTION

With more and more antibiotics occurring in sorts of ecosystems, it has been realized that the antibiotics dispersed in the environment may disturb the original microbial community and accelerate the evolution of species (Hirsch et al. 1999; Giger et al. 2003; Batt et al. 2006). So far, there are various studies about the microbial communities with impaction of antibiotic residues, e.g. in soils (Westergaard et al. 2001; Zielezny et al. 2006; Hammesfahr et al. 2008) and aquatic sediments (Naslund et al. 2008; Suzuki et al. 2008). However, those reports are restricted in the non-industrial environments, and the knowledge about the bacterial community in the engineering biomass (activated sludge or biofilm) designed to treat antibiotics in wastewater plants is still a blank.

The performance of the biofilms developed on granular activated carbon (GAC) for treating ampicillin (AMP)-bearing wastewater has been presented in Chapter 4. Therefore, this chapter aims to examine and analyze the bacterial community of the biofilms developed for AMP treatment through advanced molecular biology techniques, such as PCR-DGGE and 16S rRNA gene sequencing, with focus on the tendency of the community changes along with the AMP removal performance. It is expected that this study can offer useful information for further optimization of the biofilm process towards the high-efficiency treatment of antibiotic-bearing wastewater.

5.2 MATERIALS AND METHODS

5.2.1 Biofilm Samples

The operation conditions of R1 to R3 were described in Chapter 4. R1 served as the control free of AMP, while R2 and R3 were supplemented with AMP at the concentrations of 4 and 8 mg L^{-1}, respectively. The biofilm samples were periodically collected from each reactor in the course of the operation. For the purpose of comparison, suspended sludge was cultured in a sequencing batch reactor (SBR) fed with the synthetic wastewater (Chapter 4), while supplemented with 8 mg L^{-1} AMP.

5.2.2 Extraction of DNA from Biofilm

BGAC samples from R1 to R3 were resuspended in 10 mL of deionized water and vortexed for 5 minutes. The collected detached biofilms and the suspended sludge cultivated in SBR were used for the DNA extraction using the SoilMaster™ Kit (Epicentre, Madison, US) according to manufacturer's instructions. After a series of carefully rinsing, the DNA was eluted with 100 µL TE buffer (10 mM Tris–HCl at pH 7.5, 1 mM EDTA). DNA purity and yield were measured with a biophotometer (Eppendorf, Germany). The extracted DNA was stored at -20°C before use.

5.2.3 Amplification of 16S rDNA Fragments by PCR

For each sample, replicate DNA extracts were pooled for polymerase chain reaction (PCR) amplification, using GoTaq® Green Master Mix Kit (Promega, Madison, US) and primers with the following sequences: 5'-ATTACCGCGGCTGCTGG-3' and 5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACG

GGAGGCAGCAG-3’. The second primer contains a 40 bp GC clamp at its 5’ end. The primers amplify the V3 region of the bacterial 16S rDNA of corresponding to position 341 to 534 in *E. coli* (Muyzer et al. 1993). A touchdown PCR program was employed in the GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA): 1 minute at 95°C, 1 minute at 65°C, 2 minutes at 72°C with a touchdown of 0.5°C per cycle for the first 20 cycles, and followed by another 15 cycles at the annealing temperature of 55°C. A final extension was performed at 72°C for 6 minutes.

5.2.4 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was performed with a D-Code Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, USA). 40 μL of the PCR products were loaded on an 8% acrylamide gel with a denaturing gradient of 30-70% (where 100% denaturant was a mixture of 7 M urea and 40% formamide). The gel was pre-run at 85 V and 60°C in 1× TAE for 30 minutes. The samples were then loaded and the gel was run at 85 V for 15 hours. Gels were stained in 50 μg mL^{-1} ethidium bromide for 15 minutes and then destained with 1× TAE for 30 minutes before photographed by the Gel Viewer System Kodak EDAS 290 (Tokyo, Japan).

5.2.5 Numerical Analysis of DGGE Profiles

The digitalized images of DGGE profiles were analyzed with the Quantity One software, Version 4.6.2 (Bio-Rad Laboratories, Hercules, USA). In the DGGE analysis, the seed activated sludge was used as the “external reference pattern” for patterns alignment and comparison (Eichner et al. 1999). Each band was characterized by its position and its relative intensity in the profile, which could be calculated by the relative surface of the peak in the profile. Lastly, these data were used for diversity analysis (Fromin et al. 2002).

5.2.5.1 Computation of diversity

Based on number and intensity of DGGE bands, the following indices can be calculated for characterization of the diversity of the biofilm community.

(1) Richness (R) (Fromin et al. 2002)

The total number of species represented in community (richness) is evaluated from the index R, referring to total different DGGE bands.

(2) Shannon's diversity index (H) (Shannon 1948)

Shannon index, also called Shannon-Weaver diversity index, is a composite index integrating both richness and evenness. It is the most popular and direct measure of the community diversity (Stocker et al. 1985; Tay et al. 2005; Li et al. 2008; Andreote et al. 2009).

$$H=-\sum p_i \ln p_i \qquad (5.1)$$

in which p_i is the relative intensity of band i.

(3) Pielou's evenness index (E) (Pielou 1966)

The way by which individuals are distributed among the community is called evenness reflected by E:

$$E=H/\ln R \qquad (5.2)$$

in which H is the Shannon-Weaver index in Eq. 5.1. Pielou's index is equal to 1 for completely even distribution of species.

(4) Simpson dominance index (D) (Begon et al. 1990)

Simpson index is calculated as follows:

$$D=\sum p_i^2 \qquad (5.3)$$

For a given richness (R), dominance (D) increases with evenness (E), whereas for a given E, D increases with R.

5.2.5.2 Computation of similarity

As each bacterial community presents a unique band pattern, similarities between band patterns taken in pairs can be expressed as a percentage of a similarity coefficient. The method for determination of similarity in Quantity One is the Dice Coefficient (Dice 1945), and a dendrogram can be generated from the cluster analysis of similarity matrix.

In this study, nonmetric multidimensional scaling (NMDS) was introduced to show the changes of bacterial community during the biofilm development. The DGGE band patterns were converted to a binary matrix to make the data accessible to statistical analysis. The presence or absence of a nucleic acid band at the same height in each lane was marked with a 1 or 0. Based on this binary matrix, a distance matrix was then generated using the SPSS software, Version 11.5 (SPSS Inc., Chicago, USA). As the result, a map of every community structure at a given time can be created, and the data were presented in a Euclidean plane in which highly similar measurements are aggregated closely. The dimensions (axes) in the map have no special significance and can be rotated or mirrored without influencing the reproduced distances to the observed distances. NMDS has been proven to be a useful tool for analysis of DGGE banding patterns (Van Hannen et al. 1999; Miura et al. 2007).

5.2.6 Amplification and Purification of 16S rRNA Gene

Amplification of the 16S rRNA gene was carried out using the Bacteria-specific primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and the universal primer 1492R (5'-GGTTACCTTGTTACGACTT-3') (Labrenz et al. 2004). The two primers amplify nearly the full length of the 16S rRNA gene. All reagents, except the primers and template DNA, came from the Taq PCR Core Kit (Qiagen, Duesseldorf, Germany). The PCR reaction set up were as follows: 5.0 µL of 10× PCR Buffer, 2.0 µL of $MgCl_2$, 1.0 µL of dNTP, 0.125 µL of each forward and reverse primers, 0.25 µL of Taq polymerase, 4.0 µL of DNA template and 42.5 µL of Milli-Q water, making up a total of 55.0 µL reaction volume. The cycling conditions were presented as follows: initial denaturation at 95°C for 15 minutes, followed by 16 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes with a final extension of 72°C for 20 minutes.

Purification of the 16S rRNA gene was carried out either using the QIAquick PCR Purification Kit (Qiagen, Duesseldorf, Germany), or the QIAquick Gel Extraction Kit (Qiagen, Duesseldorf, Germany) according to the manufacturer instructions. When additional non-target bands were detected by agarose gel electrophoresis, gel extraction was conducted to excise the desired band. Otherwise, the QIAquick PCR Purification Kit was used to remove excess primer dimers. Purified DNA was eluted out from the spin columns with 40 µL of Milli-Q water in either case. In addition, spectrophotometric measurements and agarose gel electrophoresis were carried out after purification to ensure sufficient concentration and purity of eluted PCR products.

5.2.7 Cloning of 16S rRNA Gene

The 16s rRNA gene was amplified from total genomic DNA and purified as described above. Purified PCR products were used to construct the 16s rRNA gene clone library using the pGEM-T Easy Vector System (Promega, Madison, USA). Ligation of the purified PCR product to the pGEM-T vector was carried out at 4°C overnight with a vector to insert molar ratio of 1:3, and was transformed into chemically competent JM109 cells by heat shock at 42°C for 45 to 50 seconds.

100 µL transformation cultures were plated onto duplicate selective Luria-Bertani (LB) plates and incubated at 37°C overnight. Selective LB plates contained 100 µg mL^{-1} of AMP. 20 µL of 50 mg mL^{-1} molecular grade X-Gal (Sigma-Aldrich, Singapore) dissolved in molecular grade DMSO (Sigma-Aldrich, Singapore) with 100 µL of 100mM IPTG (Difco Laboratories, Ann Arbor, US) were spread over the surface of the plates with 30 minutes incubation at 37°C for blue-white screening. 100 white or pale blue colonies were randomly selected and sterile disposable inoculating loops were used to inoculate the selected colonies into 5 mL of selective LB broth containing 100 mg L^{-1} AMP and incubated at 37°C overnight.

5.2.8 Sequencing of 16S rRNA Gene

Purified 16S rRNA gene was sent to a local research lab for sequencing, with the forward primer 27F as the primer. Similarity searches were performed using the BLAST algorithm at GenBank (http://www.ncbi.nlm.nih.gov/) as well as SEQMATCH at the Ribosomal Database Project (RDP-II) (http://rdp.cme.msu.edu/html/) to affirm the identity of the isolates. The nucleotide sequences reported in this study were deposited in the GenBank database with the following accession numbers: GU560157 to GU560185, GU294846 to GU294853, and GU319967 to GU319971.

5.3 RESULTS

5.3.1 Diversity Analysis of DGGE Profiles

DGGE represents an efficient approach for study of the diversity of different microbial communities in various environments (Muyzer 1999). In DGGE, DNA fragments of the same length, but with a different base-pair sequences can be separated according to melting properties, and a band then represents a genus or specie of microbe (Yang et al. 2009).

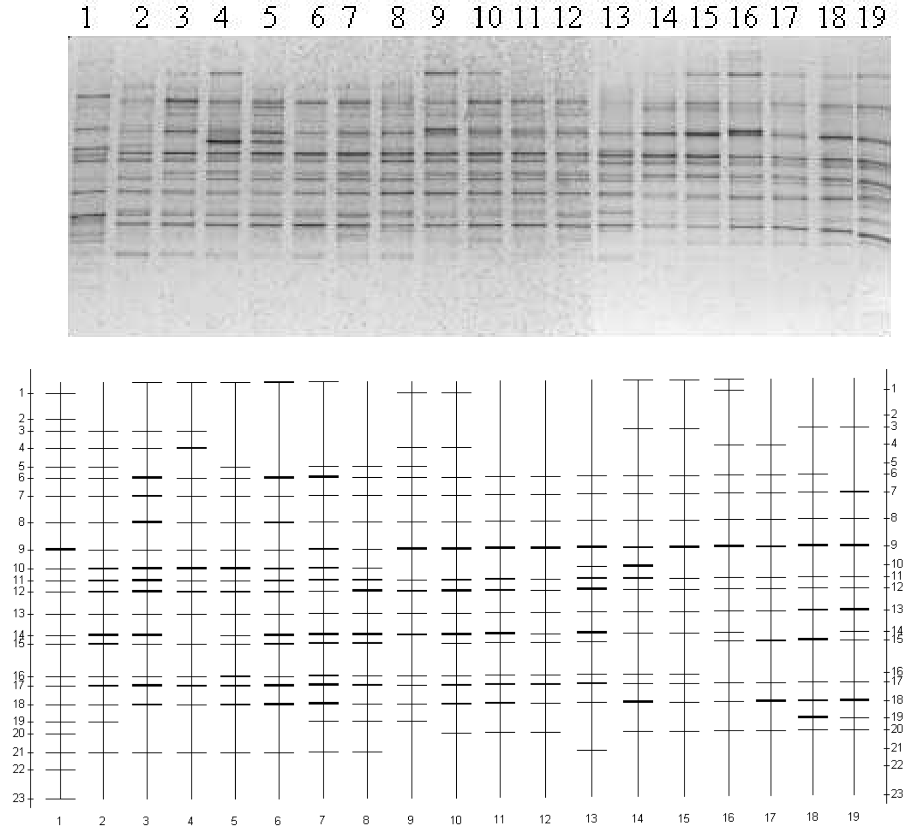


Figure 5.1 DGGE profiles of the biofilm communities. Lane 1: seed sludge; lanes 2-7: R1 biofilms on day 4, 18, 24, 31, 38 and 51; lanes 8-12: R2 biofilms on day 4, 24, 31, 38 and 51; lanes 13-19: R3 biofilms on day 4, 10, 18, 24, 31, 38 and 51, respectively.

Fingerprint patterns of the DGGE profiles of the biofilms developed in R1 to R3 in the course of operation are shown in Figure 5.1. The seed sludge (Lane 1) was used as the external reference (Eichner et al. 1999) to align the band patterns for the patterns comparison of different gels with each other. In this study, after alignment and normalization, the same sample on different gels had a similarity over 95%.

According to the DGGE profiles and definition formula given in Section 5.2.5.1, the indices were calculated to evaluate the diversity of the bacterial community in the biofilms (Figure 5.2).

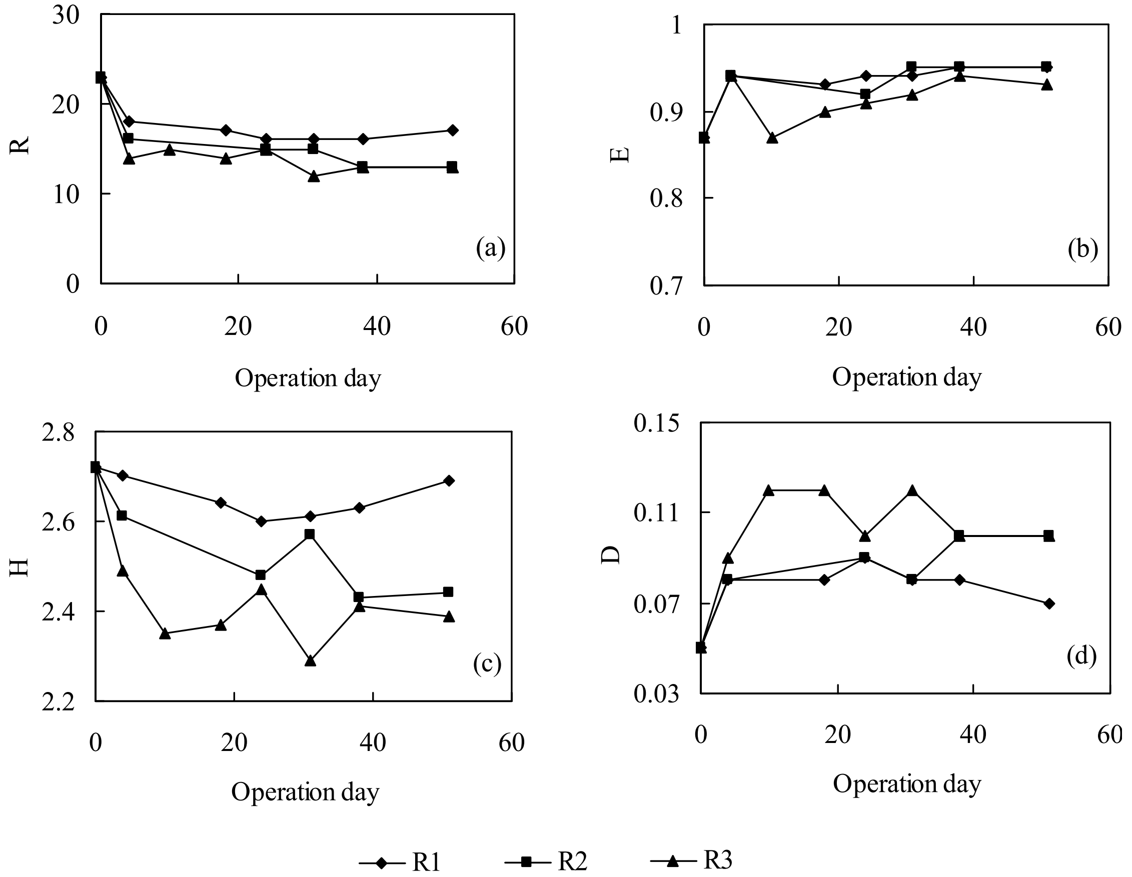


Figure 5.2 Diversity indices based on DGGE profiles over the biofilm development process. (a) Richness index (R); (b) Pielou's evenness index (E); (c) Shannon's diversity index (H) and (d) Simpson dominance index (D).

In Figure 5.2, Richness index (R) and Shannon's index (H) in bacterial communities fed with AMP (R2 and R3) were lower than that without AMP (R1), implying that the biofilms developed with presence of AMP had lower diversity than that without AMP. Pielou's index (E) for each reactor was found to be close to 1, indicating a very homogeneous community in all the biofilm samples. Moreover, the small variation of E in R2 and R3 showed that the AMP impact on the evenness of the biofilm community was negligible. Simpson index (D) shows the weight of dominant species in the community. The average Simpson index increased with the increase in the AMP concentration from 0 to 8 mg L^{-1} in R1 to R3. This suggests that certain resistant species could survive and thrive with the existence of AMP.

5.3.2 Cluster Analysis of DGGE Profiles

Dendrogram

Banding patterns generated by DGGE for the R1 to R3 biofilms sampled on different operation days were used for cluster analysis. A dendrogram (Figure 5.3) with all these samples was constructed through the unweighted pair-group method using arithmetical averages (UPGMA). Obviously, the upper cluster included all samples from R1 plus one from R2 on day 4 (# 8) and one from R3 on day 10 (# 14), i.e. only at the initial stage (first two weeks), the settled communities were similar in all these three reactors. However, R2 and R3 communities clearly constituted a cluster more distantly related to the samples from the blank R1. This implies that AMP would have a substantial effect on the bacterial community structure.

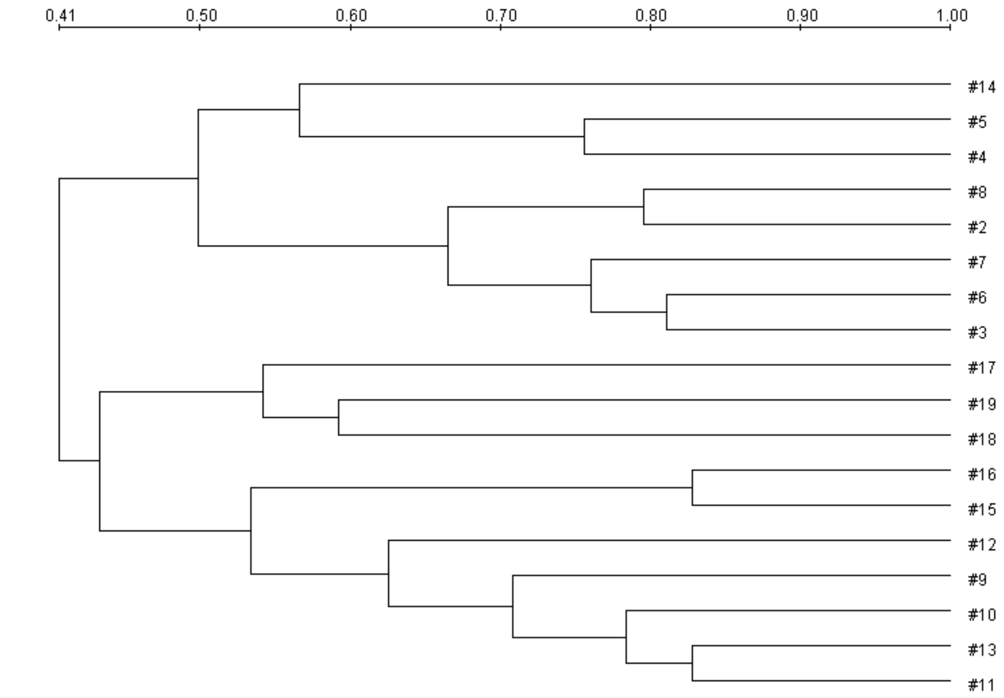


Figure 5.3 Cluster analysis of the DGGE profile by UPGMA method. # 2-7: R1 biofilms on day 4, 18, 24, 31, 38 and 51; # 8-12: R2 biofilms on day 4, 24, 31, 38 and 51; # 13-19: R3 biofilms on day 4, 10, 18, 24, 31, 38 and 51, respectively.

NMDS Map

To understand the effect of AMP on the alteration of bacterial community structures, DGGE banding patterns were analyzed by non-metric multidimensional scaling (NMDS) approach (Figure 5.4). In the NMDS plots, the distance between the points reflects the similarity between different DGGE profiles. Similar community structures are clustered closely. Hence, the variation degrees of the bacterial community can be compared among different sites.

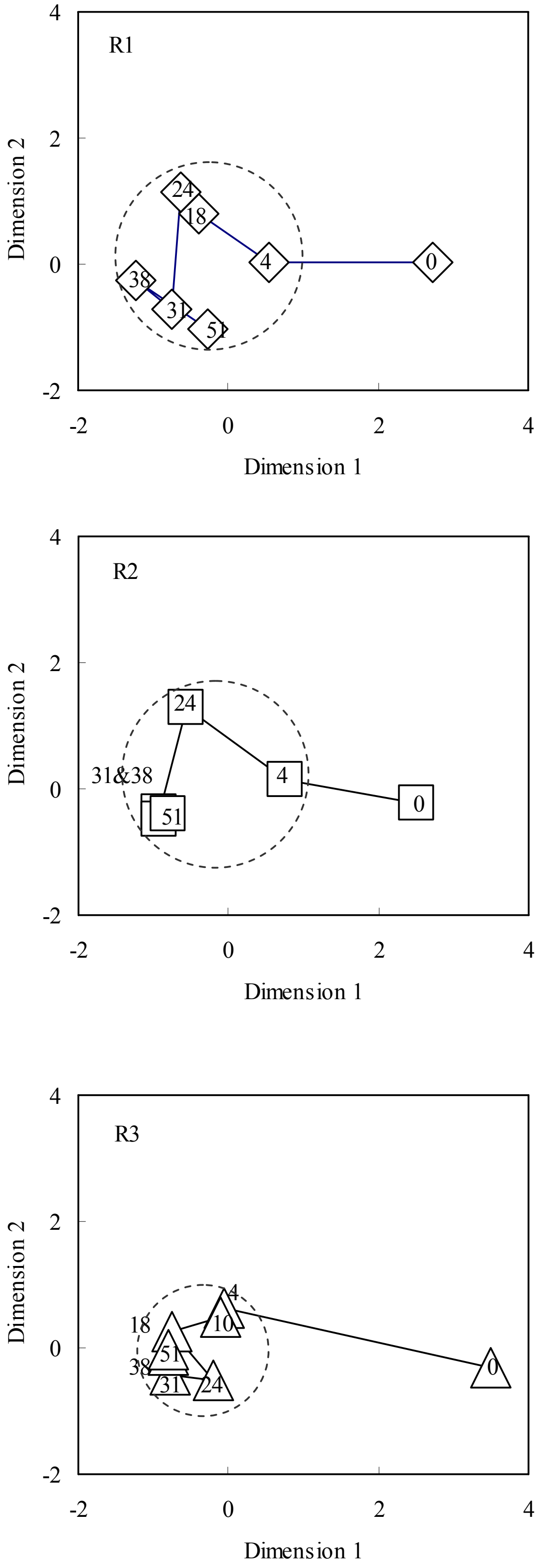


Figure 5.4 NMDS plots of the R1 to R3 biofilms. The number inside the symbol refers to the operation days of each reactor, except that “0” refers to the seed sludge.

In Figure 5.4, one point represents the community structure on a specified operation day, and the distance between the two points reflects the similarity of microbial community structures. Thus, the communities with more similar microbial structures would be aggregated more closely. Relatively large variations in the microbial communities were observed within the first four days of operation in R1 to R3. From day 4 onwards, the community changes in the biofilms developed in R2 and R3 supplemented with AMP became less obvious than those observed in R1 free of AMP. These in turn imply that the community structure should become more stable than the counterpart biofilm without exposure to AMP after the biofilms adapted to AMP stress.

5.3.3 Identification of Microbial Species

It has been known that the biomass in the biological wastewater treatment process contains various types of microbial species, most of which are viable, but non-culturable (Grady et al. 1999), meaning that the conventional culture-based method has its own limits for the full screening of the bacteria in the biofilm or activated sludge. To date, advanced molecular biology techniques have been exploited for the study of non-culturable as well as culturable microbes, e.g. cloning and sequencing 16S ribosomal RNA encoding genes (Hugenholtz et al. 1998).

5.3.3.1 Bacteria in biofilms developed with/without AMP

To study temporal changes of the bacterial populations, the biofilm samples from R3 were examined on day 10, 24 and 38 (Tables 5.1 to 5.3). It appears from the biofilm growth curve (Figure 4.2) that these three samples indeed represent the initial biofilm (day 10), the growing biofilm (day 24) and the mature biofilm (day 38), respectively.

Table 5.1 Closest relative to species identified by sequencing 16S rRNA gene for the initial biofilm developed with 8 mg L^{-1}AMP on day 10.

Representative clone	No. of clones in OTU*	Closest relative	Similarity	Accession number
R3W2S84	17	*Acidovorax sp.*	99%	EU375647.1
R3W2S47	13	*Acinetobacter sp.*	99%	EF111219.1
R3W2S82	6	*Flavobacterium kamogawaensis*	95%	AB275999.1
R3W2S90	6	*Azoarcus sp.*	100%	DQ851175.1
R3W2S96	4	*Pseudoxanthomonas sp.*	99%	EF540482.1
R3W2S6	2	*Acinetobacter sp.*	99%	EU037279.1
R3W2S55	2	*Acinetobacter johnsonii*	100%	EF114343.1
R3W2S4	1	*Acidovorax facilis*	99%	EU730927.1
R3W2S25	1	*Sphingobium sp.*	99%	AY689029.1
R3W2S44	1	*Thauera aromatica*	99%	AJ315680.1
R3W2S58	1	*Delftia sp.*	100%	FJ378038.1
R3W2S89	1	*Pelomonas saccharophila*	99%	AM501432.1
R3W2S91	1	*Stenotrophomonas sp.*	99%	FJ357297.1

*OTU: operational taxonomic unit

Table 5.2 Closest relative to species identified by sequencing 16S rRNA gene for the growing biofilm developed with 8 mg L^{-1} AMP on day 24.

Representative clone	No. of clones in OTU*	Closest relative	Similarity	Accession number
R3W4S4	27	*Acidovorax sp.*	99%	Y18617.1
R3W4S52	16	*Pseudoxanthomonas mexicana*	99%	FM213381.2
R3W4S9	5	*Azoarcus sp.*	96%	AM406670.1
R3W4S11	2	*Aquimonas voraii*	98%	AY544768.1
R3W4S63	2	*Bacterium SG-3 16S*	99%	AF548381.1
R3W4S1	1	*Pseudomonas anguilliseptica*	99%	AF439803.1
R3W4S15	1	*Delftia acidovorans*	99%	CP000884.1
R3W4S16	1	*Sphingomonas sp.*	98%	AB047364.1
R3W4S19	1	*Acinetobacter sp.*	99%	EF494200.1
R3W4S45	1	*Sphingomonas sp.*	98%	AY947554.1
R3W4S47	1	*Thauera butanivorans*	98%	AB021377.1
R3W4S56	1	*Aquabacterium sp.*	98%	EF179861.1
R3W4S68	1	*Rhodocyclus sp.*	99%	AY691423.1
R3W4S69	1	*Leadbetterella byssophila*	95%	AY854022.2
R3W4S71	1	*Rhizobium sp.*	99%	AM084043.1

*OTU: operational taxonomic unit

Table 5.3 Closest relative to species identified by sequencing 16S rRNA gene for the mature biofilm developed with 8 mg L^{-1} AMP on day 38.

Representative clone	No. of clones in OTU*	Closest relative	Similarity	Accession number
R3W5S3	14	*Acidovorax sp.*	99%	Y18617.1
R3W5S36	6	*Pseudoxanthomonas mexicana*	99%	FM213381.2
R3W5S8	4	*Azoarcus sp.*	96%	AM406670.1
R3W5S1	2	*Aquimonas voraii*	98%	AY544768.1
R3W5S6	2	*Rhodobacter sp.*	96%	AB251408.1
R3W5S29	2	*Hydrogenophaga sp.*	98%	AB166886.1
R3W5S33	2	*Rhodobacter sp.*	97%	AM888193.1
R3W5S10	1	*Flavobacterium gelidilacus*	95%	EU090722.1
R3W5S17	1	*Stenotrophomonas sp.*	98%	AM745261.1
R3W5S20	1	*Acinetobacter sp.*	98%	EU337120.1
R3W5S23	1	*Rhizobium sp.*	96%	AM084043.1
R3W5S32	1	*Bacterium SG-3 16S*	99%	AF548381.1
R3W5S42	1	*Pseudomonas sp.*	99%	DQ337559.1
R3W5S43	1	*Zoogloea sp.*	99%	DQ342276.1

*OTU: operational taxonomic unit

For the purpose of comparison, bacterial species in the biofilm developed without AMP (in R1) on day 10 were also examined (Table 5.4).

Table 5.4 Closest relative to species identified by sequencing 16S rRNA gene for the biofilm developed without AMP on day 10.

Representative clone	No. of clones in OTU*	Closest relative	Similarity	Accession number
R1W2S84	17	*Pseudomonas sp.*	100%	FJ434132.1
R1W2S47	13	*Serratia sp.*	99%	GQ367571.1
R1W2S82	6	*Serratia marcescens*	92%	AJ233431.1
R1W2S90	6	*Pseudomonas lundensis*	99%	EU434368.1
R1W2S96	4	*Pseudomonas fluorescens*	98%	DQ335990.1
R1W2S6	2	*Pseudomonas syringae*	97%	AE016853.1
R1W2S55	2	*Aeromonas salmonicida subsp. salmonicida A449*	98%	DQ074967.1
R1W2S4	1	*Acinetobacter sp.*	96%	EU037279.1
R1W2S25	1	*Chromobacterium violaceum*	99%	AE012865.1
R1W2S58	1	*Pseudomonas putida*	100%	AB110608.1
R1W2S89	1	*Bacillus sp.*	99%	EF093131.1

*OTU: operational taxonomic unit

Sequences that had 97% similarity were assigned to operational taxonomic units (OTUs) with the furthest neighbour method using the DOTUR software (Schloss and Handelsman 2005). The first columns in Tables 5.1 to 5.4 show the representative clones of each OTU, while the second column represents the number of sequences in each OTU. It was found that all the representative clones had a high similarity (≥95%) to the sequences reported in the NCBI database, except for one clone in the biofilm developed without AMP on day 10 (Table 5.4) had low percentage similarity of 92% to the closest relative.

5.3.3.2 Bacteria in suspended sludge cultured with AMP

The suspended sludge cultured with AMP in the SBR was analyzed for determination of the bacterial populations on day 10 (Table 5.5).

Table 5.5 Closest relative to species identified by sequencing 16S rRNA gene for the suspended sludge cultured with 8 mg L^{-1} AMP on day 10.

Representative clone	No. of clones in OTU*	Closest relative	Similarity	Accession number
W2L85	55	*Acinetobacter sp.*	99%	EU037279.1
W2L16	3	*Acidovorax sp.*	99%	EU375647.1
W2L20	2	*Flavobacterium sp.*	94%	AM888191.1
W2L12	2	*Pelomonas saccharophila*	99%	AM501432.1
W2L60	2	*Pseudomonas sp.*	99%	FJ434132.1
W2L35	1	*Pseudoxanthomonas sp.*	99%	EF540482.1
W2L5	1	*Sphingobium yanoikuyae*	98%	AY047219.1
W2L36	1	*Shinella granuli*	99%	EU308118.1
W2L92	1	*Brevundimonas sp.*	100%	AJ227797.1

*OTU: operational taxonomic unit

BLAST search was performed on all the sequences, and all the samples were successfully identified with high similarity (>90%) (Tables 5.1 to 5.5). It can be seen that for most of the identified bacteria, the similarity scores were in between 99% and 95%. According to the criteria for identification proposed by Bosshard et al. (2003), a 95% similarity in 16S rRNA gene sequence could suggest that the clone belongs to the same genus, but may be a novel species that has not yet been identified, meaning that the 16S rRNA gene of the clone can help to identify both culturable and non-culturable species that have not been characterized yet. This indeed reflects the advantage of the molecular biology approach over the traditional culture-based method for bacteria identification.

5.4 DISCUSSION

5.4.1 Effect of AMP on Bacterial Community Diversity

Biofilm is a mixture of bacteria in which many factors may affect the structure and species composition of the microbial community. In this study, the major selective pressure is the low-concentration antimicrobial agent AMP. According to the similarity analysis (Figure 5.3), DGGE patterns of the biofilm developed with AMP gathered into one main cluster, whereas the rest DGGE patterns of the biofilm developed without AMP were grouped into another cluster. These two clusters only had about 45% of similarity. This reflects the substantial impact of AMP on the biofilm community. Furthermore, according to the band number and Shannon index, it was found that the community of the biofilm developed with AMP had less richness and lower diversity than that of the biofilm without AMP (Figures 5.2a and 5.2c). In contrast, the dominance indices of the biofilm developed with AMP were higher than that of the biofilm without AMP (Figure 5.2d). The less diversity and higher dominance could be attributed to the fact that AMP is a broad-spectrum β-lactam antibiotic, able to inhibit G^+ and some G^- bacteria. In fact, the similar phenomenon was reported in the soil microbial community. For example, Westergaard et al. (2001) found that the band numbers on the DGGE profile diminished, but intensity of some bands increased after the soil community was disturbed by the broad-spectrum antibiotic tylosin. In addition, Hund-Rinke et al. (2004) investigated the impact of another kind of broad-spectrum antibiotic, tetracycline on the ecosystem diversity of the agricultural soil using phospholipid-derived fatty acids (PLFA) method, and observed a significant reduction of the community diversity in the manure supplemented with 500 mg g^{-1} tetracycline after 8 weeks. Moreover, the tetracycline treated manure had a larger G^-/G^+ ratio than that of the control without tetracycline, due to a decrease of G^+ bacteria in the tetracycline treated manure. This and previous study shows that bacteria that can survive in the antibiotic-bearing environment mainly belong to G^- bacteria and they are responsible for antibiotic resistance.

5.4.2 Effect of AMP on Bacterial Community Stability

Concerning the effect of antibiotics on the bacterial community, it is important to understand the structural changes because the community structure is more sensitive to the disturbance of antibiotic, and such changes may be persistent over time (Westergaard et al. 2001). DGGE cluster analysis (Figure 5.2) revealed that the bacterial community structure of the biofilm treating the AMP-bearing wastewater was different from the counterpart fed with the AMP-free wastewater. This observation is consistent with the previous report that AMP could significantly alter the community structure of gut microbiota (Harmoinen et al. 2004). These suggest that AMP could act as a highly selective agent on some specific members of the microbial community, resulting in alterations in the community function.

NMDS plot (Figure 5.4) clearly shows the dynamics of the community structure. The stability of the bacterial community structures in the different reactors was found to increase with the increase in the AMP concentration from 0 to 8 mg L^{-1} in R1 to R3. Naslund et al. (2008) investigated the effect of ciprofloxacin on the bacterial community in marine sediment at five concentration levels of 0, 20, 200, 1000 and 2000 μg L^{-1}, and found that all the points in the NMDS plot (Figure 5.5) could be grouped into three polygons, and the tighter clustering at the two highest ciprofloxacin doses (1000 and 2000 μg L^{-1}) suggests a lower variation. Both this and previous study support the view of that the more stable microbial community would be expected at higher antibiotic concentration (e.g. AMP).

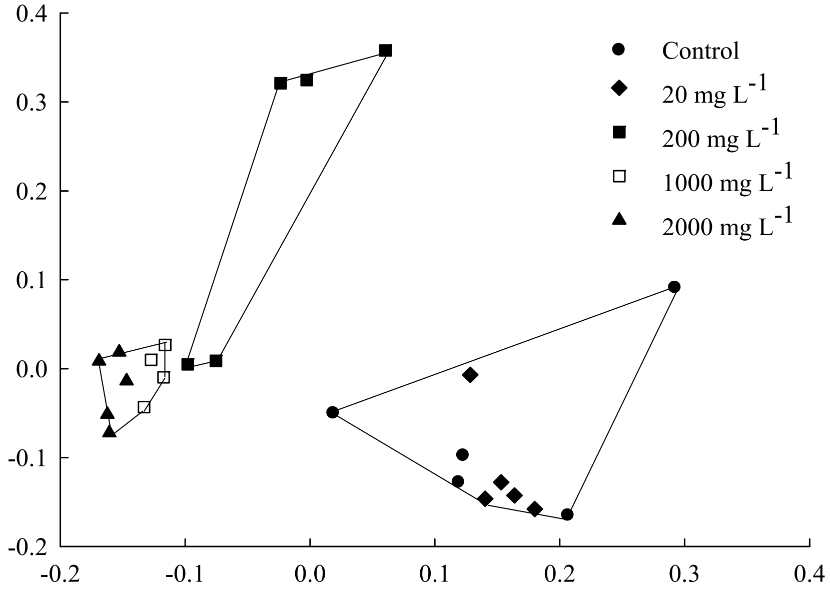


Figure 5.5 NMDS plot of the sediment communities exposed to different concentrations of ciprofloxacin after 7 weeks. Data from Naslund et al. (2008).

5.4.3 Effect of AMP on Bacterial Community Populations

5.4.3.1 Comparison of biofilm developed with/without AMP and sludge with AMP

In order to look into the characteristics of the bacterial community of the biofilm developed with AMP, it is necessary to make comparison with the controls, i.e. the biofilm developed without AMP and the suspended sludge cultured with AMP. For this purpose, biofilm with AMP, biofilm without AMP and sludge with AMP were sampled from R3, R1 and SBR on day 10 for microbiological examination. Community members of these three samples were illustrated in Tables 5.1, 5.4 and 5.5, and subsequently the bacterial species of interest are compiled in Figure 5.6, such as *Bacillus sp.*, *Azoarcus sp.*, *Acinetobacter sp.*, *Flavobacterium sp.* and *Pseudomonas sp.*.

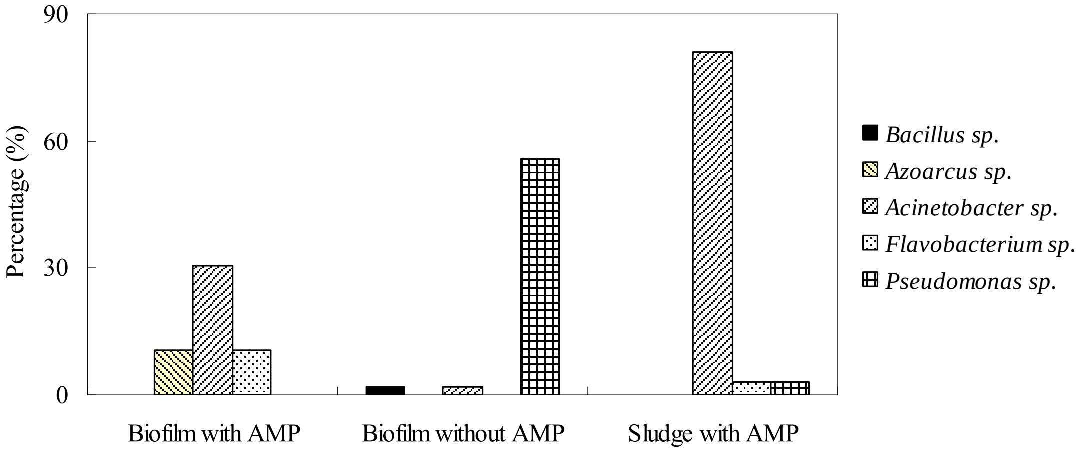


Figure 5.6 Distributions of bacteria of interest in the biofilms developed with/without AMP, and suspended sludge cultured with AMP on day 10.

It was found in Tables 5.1, 5.4 and 5.5 that all the identified bacteria were G^- bacteria except for *Bacillus sp.* which was present in the biofilm without AMP (Figure 5.6). Genus *Bacillus* is a kind of G^+, aerobic or facultative endospore-forming bacteria. Since AMP is a broad-spectrum β-lactam antibiotic able to inhibit G^+ and some G^- bacteria, this may provide an explanation of the appearance of the G^+ bacteria in the control biofilm, but not in the biofilm and sludge exposed to AMP.

Moreover, *Azoarcus sp.* was only detected in the biofilm with AMP at an abundance of 10.7%, but not in the biofilm without AMP or sludge with AMP (Figure 5.6). *Azoarcus sp.* was a kind of G^- anaerobic denitrifyer. Although resistance of *Azoarcus sp.* to antibiotics has not been extensively explored, many species and strains in this genus had been found not to be resistant to AMP (Boone et al. 2005). Therefore, the unique occurrence of this bacteria in the biofilm developed with AMP could be attributed to the underneath zone of the biofilm in which an anaerobic and protective environment would be eventually created for the survival of this species.

One of the identified species, *Acinetobacter sp.*, was found at an abundance of 30.4%, 1.9% and 80.8% in the biofilm developed with AMP, biofilm without AMP and sludge with AMP (Figure 5.6), respectively. In fact, *Acinetobacter sp.* strains have been isolated from diverse environments (Baumann 1968; Warskow and Juni 1972; Mendes et al. 2009), suggesting their universal existence in natural environments. *Acinetobacter sp.* has been commonly implicated in nosocomial infections due to its ability to rapidly develop resistance to antibiotics. Its resistance to AMP is closely associated to the production of β-lactamases (Bergogne-Berezin and Towner 1996). A study on the sewage receiving waste from a hospital and a pharmaceutical plant also suggests that *Acinetobacter sp.* is not inherently resistant to any antibiotic, but is predisposed to develop resistance against antibiotics (Guardabassi et al. 1998). A rapidly acquired AMP-resistance mechanism that is independent of the biofilm phenotype may be a factor contributing to its extreme predominance (80.8%) in the suspended sludge with AMP in the planktonic form.

It is noted in Figure 5.6 that distribution of *Flavobacterium sp.* in the biofilm developed with AMP (10.7%) is higher than that of the sludge with AMP (2.9%), and this species was not found in the biofilm without AMP. Previous study about 1011 stains of imipenem resistant *Flavobacterium* showed that *Flavobacterium spp.* were highly resistant to β-lactam antibiotics (Zhang 2007). The exclusive presence of this bacterial species in the biofilms and suspended sludge exposed to AMP further confirms its resistance to AMP, and this bacterium could be assumed to acquire AMP resistance more slowly than *Acinetobacter sp.* as evidenced by the reverse tendency of distribution for these two bacteria in the biofilm with AMP and sludge with AMP (Figure 5.6).

As shown in Figure 5.6, the abundance of *Pseudomonas sp.* was as high as 55.6% in the biofilm developed without AMP, but 0% in the biofilm with AMP and 2.9% in the sludge with AMP. Generally, *Pseudomonas sp.* is a kind of ubiquitous bacterium and is related to the degradation of a broad range of synthetic and natural organic compounds in wastewater treatment plants. In fact, *Pseudomonas sp.* has been reported to be the dominant species in many engineering microbial communities (Martiny et al. 2005; Ivnitsky et al. 2007). Based on screening of the antibiotic-resistance bacteria collected from the upstream and downstream of a wastewater treatment plant handling the penicillin production waste, Dong et al. (2009) found that *Pseudomonas sp.* existed in both sites, whereas *Acinetobacter sp.* only appeared in the effluent of the penicillin production wastewater, but not in the upstream of the plant. Therefore, it would be reasonable to consider that *Pseudomonas sp.* should be resist to AMP, but its resistance is weaker than that of the other species, like *Acinetobacter sp.*.

5.4.3.2 Comparison of different age biofilms developed with AMP

Figure 5.2 shows that the 10-day, 24-day and 38-day biofilms developed in R3 supplemented with 8 mg L^{-1} AMP may represent the biofilms at different growth stages, i.e. initial biofilm, growing biofilm and mature biofilm, respectively. Based on the clone libraries obtained (Tables 5.1 to 5.3), bacteria in the R3 biofilms at the different growth ages can be classified according to phylum/class (Figure 5.7). It was found that all the identified bacteria in the different age biofilms fell into four phylogenetic divisions, i.e. α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria and Bacteroidetes. Regardless of the biofilm age, β-Proteobacteria was found to be constantly dominant with the abundance of about 50% in the biofilm community, whereas *Acidovorax sp.* and *Azoarcus sp.* in β-Proteobacteria were the two most abundant genera in the biofilm community. For example, *Acidovorax sp.* was found in the initial biofilm, the growing biofilm and the mature biofilm at the abundance of 32%, 43% and 36%, respectively. Kraigher et al. (2008) also reported that β-Proteobacteria, mainly *Acidovorax*-related species were predominant in the activated sludge communities subject to the other non-antibiotic pharmaceuticals. The dominance of *Acidovorax sp.* in the biological wastewater treatment process should be ascribed to its strong biodegradation capability for the recalcitrant organics, such as acrylonitrile (Wang et al. 2004), chlorobenzenes (Monferran et al. 2005) and polycyclic aromatic hydrocarbon (Singleton et al. 2009). It appears from this study that *Acidovorax sp.* would also be the dominant species in the biofilms treating AMP-bearing wastewater.

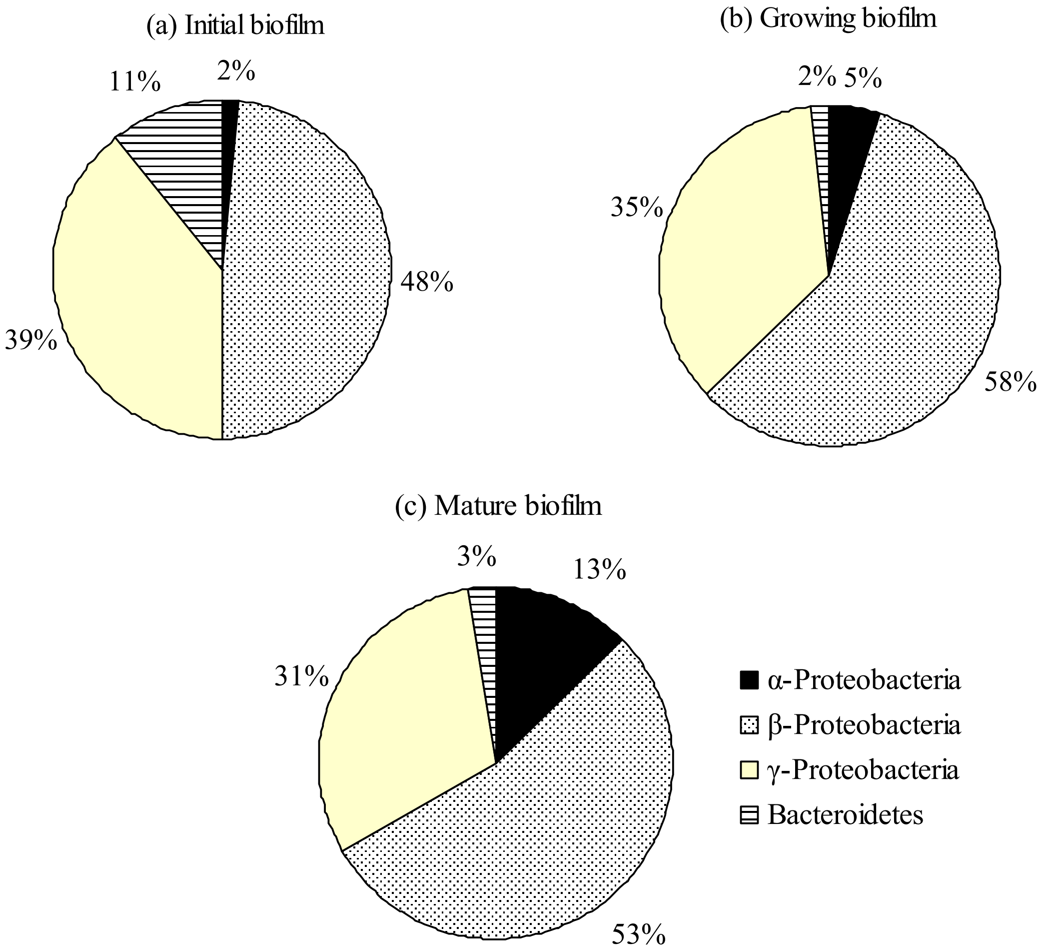


Figure 5.7 Distribution of each phylum/class division in the R3 biofilms at different growth stages.

According to the AMP resistant capability reported in previous studies, all the identified bacteria in the biofilms developed with AMP in this study (Tables 5.1 to 5.3) can be grouped into three categories: resistant, unknown and susceptible. The resistant category basically includes *Acidovorax sp.* (Wen et al. 2007; Liu et al. 2009), *Acinetobacter sp.* (Bergogne-Berezin and Towner 1996), *Flavobacterium sp.* (Zhang 2007), *Delftia sp.* (Liang et al. 2005), *Stenotrophomonas sp.* (Avison et al. 2002; Gould et al. 2006) and *Pseudomonas sp.* (Sokari et al. 1988), whereas *Azoarcus sp.* (Boone et al. 2005), *Leadbetterella sp.* (Weon et al. 2005), *Rhodobacter sp.* (Baumann et al. 1989) and *Hydrogenophaga sp.* (Yoon et al. 2008) can be grouped into the susceptible category. The rest bacteria shown in Tables 5.1 to 5.3 have unknown AMP resistant capability. Furthermore, Figure 5.8 shows the total DNA of each identified bacterial species on the GAC.

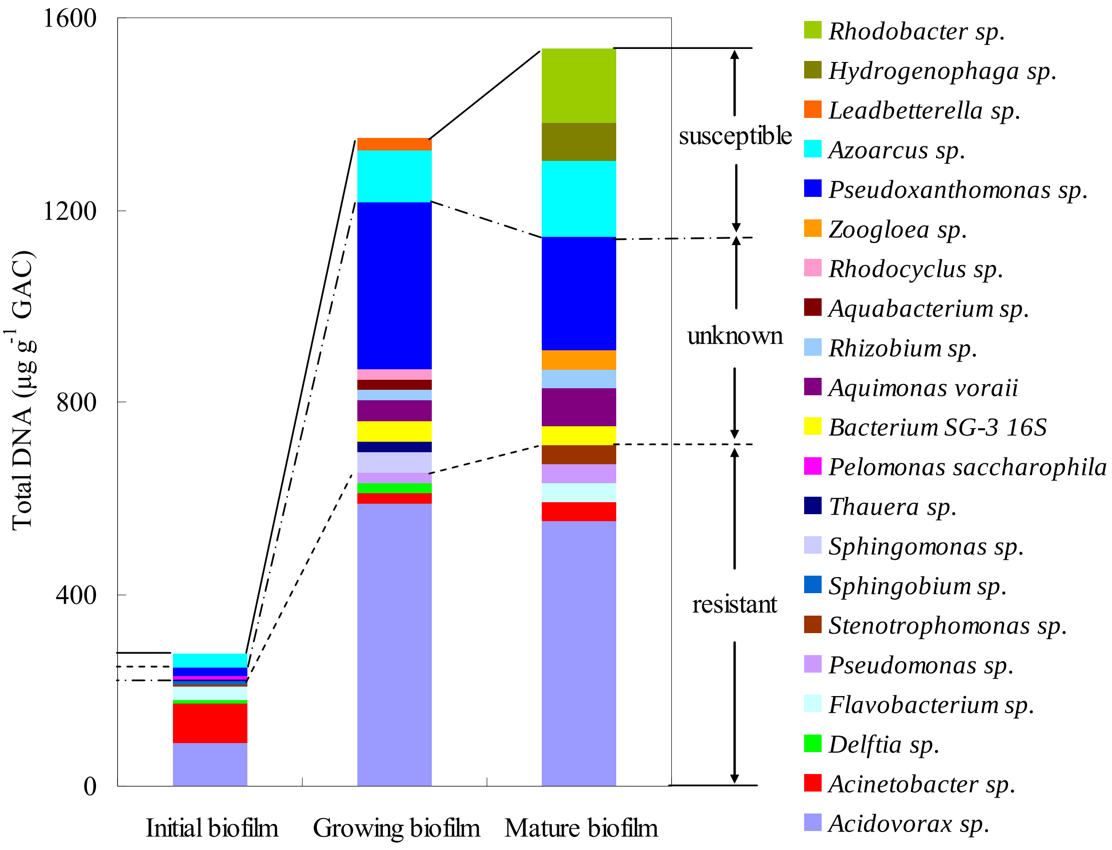


Figure 5.8 Amount of identified species in the R3 biofilms at different growth stages.

It can be seen in Figure 5.8 that *Acinetobacter sp.* was a dominant species with an abundance of 30% in the initial biofilm, but its abundance drastically decreased to less than 3% in the growing and mature biofilms. Historically, *Acinetobacter sp.* has been thought to be present in a small proportion (3-6%) in the conventional activated sludge process (Hiraishi et al. 1989). The high percentage of *Acinetobacter sp.* in the initial biofilm implies that this species would play an important role in developing the biofilm phenotype, which is most likely due to its high surface hydrophobicity and low zeta potential that facilitate the microbial attachment and subsequent coaggregation of different genera (Malik et al. 2003). Moreover, *Acinetobacter sp.* has been known to produce a variety of extracellular polymers, mainly polysaccharides (Kaplan and Rosenberg 1986), which could be the reason that the ratio of extracellular polysaccharides to extracellular proteins increased sharply when biofilm grew from the initial phase into the growing phase (Figure 4.6). After the initial build-up of the biofilm phenotype, *Acinetobacter sp.* was gradually overtaken by other bacteria with strong degradation capability of chemicals (Figure 5.8).

Obviously, bacteria with known resistant capability to AMP would have higher chance to survive in the AMP-bearing environment. However, the abundances of bacteria with unknown resistant capability to AMP and those susceptible to AMP also tended to increase from 23% in the initial biofilm, to 52% in the growing biofilm and further to 54% in the mature biofilm (Figure 5.8). Although these bacteria would not directly contribute to AMP biodegradation, they may have other biological functions for wastewater treatment. For example, *Azoarcus sp.* and *Thauera sp.* are denitrifyers responsible for wastewater denitrification (Liu et al. 2006), whereas *Rhodocyclus sp.* plays a significant role in biological phosphorus removal (Hesselmann et al. 1999). Therefore, the non-AMP-resistant bacteria with specific biological functions would also be selected by the complex wastewater environment. In addition, *Azoarcus sp.* and *Thauera sp.* are both anaerobic bacteria and are closely related to the biodegradation of aromatic compounds (An et al. 2004; Shinoda et al. 2005; Dibenedetto et al. 2006). In fact, an anaerobic interior zone has often been found in various types of biofilms (Costerton et al. 1994), which provides an ideal environment for the facultative and anaerobic bacteria to survive even under aerobic conditions.

In order to further explore the potential AMP removal meachnisms by the R3 biofilms, BGAC samples were taken from R3 on day 10, 24 and 38 for the batch biodegradation experiments as reported in Chapter 4. These results showed that the first-order rate equation is applicable for the observed AMP removal by BGAC. The k_1 reported in Chapter 4 indeed reflects how fast AMP can be removed mainly through biodegradation, i.e. k_1 could indicate the biodegradation capability of BGAC as discussed in Chapter 4. In this, Figure 5.9 shows a correlation between k_1 and the amount of resistant bacteria identified in the R3 biofilms. It cane be seen that k_1 is positively related to the amount of the AMP degrading bacteria in the biofilms developed with 8 mg L^{-1} AMP.

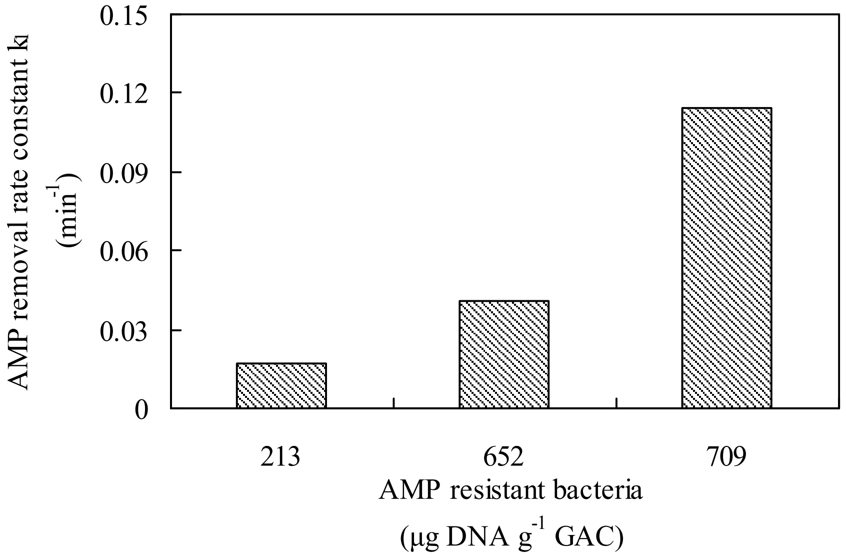


Figure 5.9 Correlation of the AMP removal rate constant and the identified resistant bacteria in the R3 biofilms on day 10, 24 and 38, respectively.

5.5 SUMMARY

It was revealed that AMP had an appreciable influence on the biofilm community by means of the advanced molecular biology techniques. The DGGE analysis suggests that the community diversity was lowered, but the community structure was more stable in the biofilms developed with AMP than in the biofilms free of AMP. The community analysis by the 16S rRNA gene sequencing shows that *Acinetobacter sp.* was dominant only in the biomass (both the biofilms and suspended sludge) exposed to AMP, implying that the AMP resistance of this bacterium could be quickly acquired through producing β-lactamases. Comparison of the different age biofilms developed with AMP further reveals that the AMP resistant bacterium *Acidovorax sp.* was the most abundant genus (about 35%) in all the three types of biofilms at different growth stages. It appears that the resistant bacteria represent the largest portion in the biofilms developed with AMP, and the increase of these bacteria would in turn help to improve the AMP biodegradation. Meanwhile, non-AMP-resistant bacteria, such as *Azoarcus sp.* and *Thauera sp.* were also found to increase on the GAC during the biofilm development due to their specific functions for treating other types of organics present in the AMP-bearing wastewater.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

This study investigated the possible removal of antibiotics by combining GAC adsorption and biodegradation in the airlift biofilm reactor with GAC as carrier. The results showed that GAC was effective for the adsorption of antibiotics. The respective maximum adsorption capacity of GAC for PCG, AMP and CPC were estimated as 427.3 mg g^{-1}, 164.2 mg g^{-1} and 33.67 mg g^{-1} at 25°C. The adsorption of these antibiotics by GAC was done through the chemical binding of the β-lactam ring with the functional groups on the GAC surface, i.e. a chemical adsorption would be involved. Langmuir isotherm provided a better description of the equilibrium data than Freundlich isotherm for all the antibiotics studied. The thermodynamic analysis ($\Delta G°$, $\Delta H°$ and $\Delta S°$) further shows that adsorption of PCG, AMP and CPC by GAC would be of the endothermic and chemisorption nature.

The theoretical origins of the widely used empirical kinetic models, i.e. first- and second-order rate equations for adsorption were explored in this study. It was demonstrated that Langmuir kinetics can be simplified to first- or second-order rate equation according to specific conditions. A general principle for simplifying Langmuir kinetics to first- or second-order rate equation was thus developed and validated by the data obtained in this study and the literature data. It was for the first time clearly shown that in what circumstance the first- or second-order rate equation would be applied for description of adsorption kinetic data.

Biofilms were successfully developed on GAC in the continuous airlift reactors supplemented with 4 and 8 mg L^{-1} AMP, respectively. It was found that AMP at the concentration levels studied would neither inhibit the biofilm formation nor alter the biofilm morphology as compared to the control reactor free of AMP. In contrast, observation in this study supports the view that subinhibitory antibiotics may promote bacterial growth and activity in some degree. Three possible mechanisms of AMP removal by BGAC were evaluated. Adsorption by biofilms was found to be negligible in the observed AMP removal by BGAC. It was revealed that the observed AMP removal was mainly attributed to the adsorption by GAC and biodegradation by the biofilms. Nevertheless, the biodegradation capability of the biofilms was gradually increased in the course of the reactor operation. As the result, bio-regeneration of the BGAC took place in the airlift bioreactor, and a sustainable operation of the system without off-line regeneration was achieved.

By means of advanced molecular biology techniques, such as DGGE and 16S rRNA gene sequencing, bacterial community of the biofilms developed on GAC for treating AMP-bearing wastewater was investigated. It was shown that AMP had a negative effect on the community diversity, but favored the community stability. Bacterial species in the community were identified and complied according to the phylogenetic classification and the AMP resistant capability. The biofilm community exposed to AMP was mainly composed of resistant bacteria able to degrade AMP, e.g. *Acidovorax sp.* and *Acinetobacter sp.* with the abundance of about 50%. Evolution of these resistant bacteria in the biofilms was found to be consistent with the variation of the AMP removal by BGAC. Consequently, this study offers in-depth insights into the treatment of the antibiotic-bearing wastewater by the combined adsorption and biodegradation.

6.2 RECOMMENDATIONS

Future work would be needed in the following aspects:

(i) Optimization of the BGAC system in terms of reactor geometry, hydrodynamic conditions and other operational parameters affecting the BGAC performance. In this study, the airlift reactor and the operation conditions were fixed in order to better understand the fate of model antibiotic and its impact on BGAC microbial community. The potential of BGAC for antibiotic removal was clearly demonstrated in this study, thus there would be a large space for engineers to further optimize the BGAC system for high-efficiency treatment of various types of antibiotic wastewaters. Such research is important for up-scaling the BGAC system.

(ii) In this study, AMP was chosen as a representative antibiotic to investigate the combined performance of adsorption and biodegradation. However, in real wastewater, different types of antibiotics would be expected, e.g. in hospital wastewater. In such a case, it is necessary to investigate the removal of multiple antibiotics by BGAC in the future study. The future study in this aspect will focus on changes in microbial degradation potential and competition of various antibiotics for adsorption.

(iii) The future study also needs to look into the microbiological nature of the identified functional bacteria in the BGAC system at the level of functional genes. Advances in genetic engineering have opened up new avenues for environmental engineers to design genetically engineered bacteria for handling special pollutants. On the top of this study, genes involving in antibiotic removal could be extracted and expreseed in other bacteria, like *E. coli*. Thus, it would be possible that BGAC might be designed with those genetically constructed microorganisms.

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