Removal of ciprofloxacin by anaerobic membrane bioreactor (AnMBR) and post-treatment of AnMBR effluent

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NANYANG
TECHNOLOGICAL
UNIVERSITY

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A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of

Doctor of Philosophy

2018

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for a higher degree to any other University or Institution.

.....

Date

.....

Student Name

This thesis is dedicated to

my dear parents, my beloved sister and brother

for being so supportive and encouraging

throughout all these years

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS	2
TABLE OF CONTENTS	3
LIST OF FIGURES	7
LIST OF TABLES	9
NOTATION	10
ABSTRACT	12
CHAPTER 1	14
Introduction	14
CHAPTER 2	18
Literature review	18
2.1 Antibiotics in the aquatic environment.....	18
2.1.1 Antibiotics and antibiotic resistance.....	18
2.1.2 Presence of antibiotics in the aquatic environment	20
2.1.3 Effect of antibiotics on aquatic organisms/human health.....	22
2.1.4 Removal of antibiotic in WWTPs	23
2.2 AnMBR for treatment of wastewater	25
2.2.1 Fundamentals of anaerobic digestion	25
2.2.2 AnMBR for the treatment of low-strength wastewaters.....	30
2.2.3 Characteristics of an AnMBR effluent	32
2.3 Post treatment of AnMBR effluents for recycling	33
2.3.1 Membranes	34
2.3.2 Advanced oxidation.....	35
2.3.3 Adsorption	38
2.4 Summary of the literature review	41
2.5 Research objectives	42
CHAPTER 3	44
Materials and Methods	44

3.1 Reactor design	44
3.2 Operation conditions for the AnMBR	46
3.3 Batch experiments	47
3.3.1 Batch experiment for anaerobic digestion	47
3.3.2 Kinetic experiment for biosorption of CIP onto AnMBR sludges	48
3.3.3 Isotherm experiment for biosorption of CIP onto AnMBR sludges.....	49
3.3.4 Batch experiment for adsorption of activated carbon in AnMBR effluent .	50
3.4 Adsorption material	50
3.5 Analytical techniques	50
3.5.1 pH	51
3.5.2 Chemical Oxygen Demand (COD)	51
3.5.3 Volatile Fatty Acids (VFAs)	51
3.5.4 Total and volatile suspended solids (TSS/VSS).....	52
3.2.5 Biogas Composition	52
3.5.6 Characterization of the activated carbons.....	52
3.5.7 Size Exclusion Chromatography (SEC)	52
3.5.8 SMP	52
3.5.9 Microbial community	53
3.5.10 CIP	54
3.5.11 CIP intermediates	54
3.5.12 Adsorption onto biomass	55
3.6 Mass balance on the removal of CIP from the AnMBR	55
3.7 Theoretical COD calculation for low-MW SMPs compounds.....	56
3.8 Biological methane potential	57
3.9 Statistical analysis.....	58
CHAPTER 4.....	59
Effects of different concentrations of CIP on anaerobic digestion.....	59
4.1 Introduction	59

4.2 Experimental and analytical details	61
4.3 Results and discussion	61
4.3.1 Effect of increasing CIP concentrations on anaerobic digestion	61
4.3.2 Removal of CIP in the batch test	73
4.4 Conclusions	75
CHAPTER 5	76
The effect of CIP on an AnMBR	76
5.1 Introduction	76
5.2 Experimental and analytical details	77
5.3 Results and discussion	77
5.3.1 Effect of CIP on the operation of an AnMBR	77
5.3.2 Effect of CIP on pH and MLSS	79
5.3.3 Effect of CIP on size distribution	82
5.3.4 Effect of CIP on low molecular weight SMPs	85
5.4 Conclusions	87
CHAPTER 6	88
Fate and removal of CIP in an AnMBR	88
6.1 Introduction	88
6.2 Experimental and analytical details	89
6.3 Results and discussion	90
6.3.1 CIP removal in AnMBR	90
6.3.2 Fate of CIP removed in an AnMBR	93
6.3.3 Biodegradation of CIP	96
6.3.4 Sorption of CIP onto biomass	98
6.4 Conclusions	101
CHAPTER 7	103
Post-treatment of AnMBR effluent by activated carbon	103
7.1 Introduction	103

7.2. Experimental and analytical details.....	105
7.3. Results and discussion.....	106
7.3.1 COD removal.....	106
7.3.2 Molecular weight distribution	108
7.3.3 VFA removal	114
7.3.4 Low-MW SMP removal	116
7.3.5 Contribution of the size fractions and specific compounds to COD removal	119
7.4. Conclusion.....	120
CHAPTER 8.....	122
Concluding Discussion, Conclusions and Future Work.....	122
8.1 Introduction	122
8.2 Concluding discussion.....	122
8.2.1 Effect of CIP on the performance of an anaerobic reactor.....	123
8.2.2 Effect of CIP on the characteristic of anaerobic effluent	123
8.2.3 Effect of CIP on microorganism.....	125
8.2.4 Removal and fate of CIP in an anaerobic reactor.....	126
8.2.5 Post treatment of AnMBR effluent by activated carbons.....	128
8.3 Conclusions	130
8.4 Recommendations for future work.....	131
REFERENCES	133
APPENDIX	154

LIST OF FIGURES

Figure 2.1	Reaction scheme for the anaerobic digestion	25
Figure 3.1	Schematic diagram of the AnMBR system	44
Figure 4.1	Effect of varying concentrations of CIP over time (day) on total methane production and relative maximum CH ₄ production rate	62
Figure 4.2	Effect of increasing concentrations of CIP over time (day) on VFA accumulation.	64
Figure 4.3	Effect of CIP on COD removal	65
Figure 4.4	Chromatograms of organic compounds found at various CIP concentrations	67
Figure 4.5	nMDS of all Operational Taxonomic Units (OTU) generated for digesters	69
Figure 4.6	The effect of CIP addition on bacteria and archaea at class level	70
Figure 4.7	CIP removal in a batch reactor with various concentrations of CIP addition	73
Figure 5.1	Effect of CIP on TSS in the different operational phase in the AnMBR..	79
Figure 5.2	Effect of CIP on the pH in the AnMBR	80
Figure 5.3	Size distribution of organic compounds in the supernatant of the AnMBR	83
Figure 5.4	Number of low-MW SMP compounds detected in AnMBR effluent with CIP added	85
Figure 6.1	Performance of AnMBR in terms of; a) COD removal, b) CIP removal efficiency, and, c) CIP adsorbed onto the sludge	91
Figure 6.2	Fate of CIP removal in the AnMBR during the addition of CIP	94
Figure 6.3	The potential intermediates for CIP biodegradation/transformation	96
Figure 6.4	Non-linear (a), Pseudo-first order (b), and Pseudo-second order (c) model fit of CIP adsorption onto AnMBR sludge.	98
Figure 6.5	Langmuir (a) and Freundlich (b) adsorption isotherms fit of CIP adsorption onto AnMBR sludge.....	100
Figure 7.1	Kinetic study of activated carbon SA2	106
Figure 7.2	COD removal by activated carbons from an AnMBR effluent	108
Figure 7.3	Size distribution of organic compounds in AnMBR effluent (EFF-II) and effluent treated by activated carbons using SEC method	111

Figure 7.4	Size distribution of the AnMBR effluent and treated effluent: (a) EFF-I, (b) EFF-III, (c) EFF-IV using a PDA detector at a wavelength of 210 nm ..	112
Figure 7.5	VFA removal by ACs in AnMBR effluents	114
Figure 7.6	COD removal by ACs from AnMBR effluents	116

LIST OF TABLES

Table 2.1	Examples of antibiotics measured in the aquatic environment. Modified from (Kümmerer, 2009)	20
Table 2.2	Advanced oxidation methods for treating anaerobic effluent	36
Table 3.1	Feed composition (for 40 gCOD/L)	45
Table 3.2	Operational condition of AnMBR during time (day)	46
Table 3.3	Characteristics of activated carbons tested	49
Table 3.4	Illustration of reactions and conversion ratios from SMP concentration to COD concentration	58
Table 4.1	Calculated fitting of experimental values to the three-parameter Gompertz equation	61
Table 4.2	Number of organic compounds found in the supernatant of the serum bottles	66
Table 4.3	Amount of CIP adsorbed onto biomass	73
Table 5.1	Overall performance of AnMBR during different operation stages	78
Table 6.1	Kinetic parameters and correlation coefficients based on the pseudo-first and pseudo-second order model	97
Table 6.2	Constants and correlation coefficients of adsorption isotherms for the adsorption of CIP onto anaerobic sludge	99
Table 7.1	Summary of the removal efficiency (%) of some major MW fractions	101
Table S4.1	List of SMPs compounds found in anaerobic digestion under CIP exposure	153
Table S7.1	The removal of some low-MW SMPs compounds by activated carbons	158
Table S7.2	List of compounds that release from activated carbon	167

NOTATION

AnMBR	Anaerobic membrane bioreactor
CIP	Ciprofloxacin
CIP-HCl	Ciprofloxacin Hydrochloride
COD	Chemical Oxygen Demand (mg/L)
CSTR	Continuously Stirred Tank Reactor
GAC	Granular activated carbon
GC-MS	Gas Chromatography coupled to Mass Spectrometry
HPLC	High Performance Liquid Chromatography
HRT	Hydraulic Retention Time
IC ₅₀	Toxic concentration that causes 50% inhibition
K _{ow}	Coefficient of Partition between Octanol and Water
LC-MS/MS	Liquid chromatography tandem-mass spectrometry
MBR	Membrane bioreactor
MLSS	Mixed Liquor Suspended Solids
MLVSS	Mixed Liquor Volatile Suspended Solids
MW	Molecular weight
PAC	Powdered activated carbon
RNA	Ribonucleic acid
SEC	Size Exclusion Chromatography
SMP	Soluble Microbial Products
SPE	Solid Phase Extraction
SRT	Sludge retention time

TOC	Total Organic Carbon
TSS	Total Suspended Solids
UASB	Upflow Anaerobic Sludge Blanket Reactor
UV	Ultra-violet
VFA	Volatile Fatty Acids (mg/L)
VSS	Volatile Suspended Solids

ABSTRACT

The presence of antibiotics has been commonly reported in the aquatic environment worldwide, and may have a substantial impact on ecosystems and human health. Of all the antibiotics present in the environment, ciprofloxacin (CIP) has sometimes been reported at extremely high concentration, in the range of mg/L. CIP is also one of the antibiotics that poses a high ecological risk, and genotoxicity and the transfer of resistance between different bacterial species were induced at concentrations as low as a few $\mu\text{g/L}$. Wastewater treatment plants (WWTP) are an important process to prevent the release of antibiotics into the environment, and of all the treatment processes available, the anaerobic membrane bioreactor (AnMBR) has considerable potential for treating wastewater, and can prevent the loss of biomass inhibited by antibiotics. However, there is a lack of data available in the literature on how effective AnMBRs are in removing antibiotics, as well as the effect antibiotics have on anaerobic digesters in general, and on the AnMBR specifically. Hence, the overall aim of this work was to explore the effect of CIP on the operation of an anaerobic batch reactor and AnMBR, and then investigate the mechanisms of CIP removal and the post-treatment of AnMBR effluent by adsorption, with the main aim of removing soluble microbial products (SMPs) and CIP. The results showed that CIP significantly inhibited the anaerobic digestion of organic matter and methanogenic activities, and volatile fatty acids (VFAs) accumulated at 0.5–50 mg/L of CIP, with the higher CIP concentration causing the strongest effect, while at 0.05 mg/L no statistical effect was observed. Furthermore, ≥ 0.5 mg/L of CIP increased low molecular weight soluble microbial products and various aromatic compounds, with p-cresol and nitrogen-containing compounds (N-compounds) dominating. *Syntrophobacter* and *Methanothrix* associated with acetogenesis and acetoclastic methanogenesis, respectively, were underrepresented in the CIP-exposed communities. However, when fed continuously to an AnMBR, no effect on performance was observed with the long-term (41 days) addition of CIP at concentrations of 0.5 ± 0.1 mg/L, and during the transition state (11 days) at concentration of 1.5 ± 0.1 mg/L. At 4.7 ± 0.7 mg CIP/L, the performance of the AnMBR was reduced with lower COD removal and biogas production. In addition, VFA and SMP accumulation, biomass reduction, and an increase in pH were also observed. More Nitrogen containing compounds (N-compounds) and esters were found under CIP exposure, and p-cresol dominated the SMPs present when high CIP concentrations were

added, with p-cresol concentrations as high as 49 $\mu\text{g/L}$. However, the overall performance of the AnMBR was still reasonable with an average of $78 \pm 13\%$ COD removal, and 50-76% of the CIP was removed when the CIP was fed at a concentration $<1.5 \text{ mg/L}$. Biological degradation was the main mechanism for removing CIP, with a few intermediate compounds detected, but when CIP was added to the feed at $4.7 \pm 0.7 \text{ mg/L}$, the removal of CIP decreased to $< 20\%$. Activated carbon adsorption was found not to be very effective for the post-treatment of AnMBR effluents, and many fractions/compounds were shown not to be readily adsorbed; both the size fractions $< 0.1 \text{ kDa}$, and about 134 kDa were difficult to remove. The identification of specific compounds in the effluent showed an excellent removal of N-compounds, phenolic compounds and CIP; in contrast, alkanes, alkenes, and esters were hard to remove.

CHAPTER 1

Introduction

Since the first antibiotic, Penicillin, was initially discovered and produced in the early 1940s, antibiotics have been used widely and saved millions of human and animal's lives from bacterial infections. It is estimated that approximately one to two hundred thousand tons of antibiotics are consumed globally (Wise, 2002). However, at the same time antibiotics were discharged into wastewater through excretion, entering wastewater treatment plants (WWTPs), and consequently discharged into the aquatic environment. The concentration of antibiotics in municipal WWTPs is normally from 0.1-0.2 $\mu\text{g/L}$, whereas in pharmaceutical manufacture and hospital WWTPs this concentration is a lot higher, from 0.5-35 and 0.5-5 $\mu\text{g/L}$, respectively (Sim et al., 2011). After treatment various antibiotics are still present and are discharged into the environment, leading to the presence of antibiotics in the worldwide environment (Kümmerer, 2009, Michael et al., 2013), and the presence of antibiotics in the aquatic environment may have a potential impact on ecosystems (Kümmerer, 2003). Moreover, it was shown that more than 80% of fecal samples in healthy people contain drug resistant organisms (Reinthal et al., 2003); thus, raw sewage usually contains antibiotic and antibiotic-resistant organisms. Antibiotic resistance is the genetic ability of specific microorganism to resist the effect of certain antibiotics, thereby reducing or eliminating the efficacy of drugs. The resistance of microorganisms to antibiotics was discovered as soon as antibiotics were discovered, however, and it was also shown that antibiotic resistance is strongly connected to antibiotic consumption (Goossens et al., 2005). With the presence of antibiotics in the aquatic environment, the risk of spreading the resistance genes increases, and this causes an increasing risk to human health (Fent et al., 2006, Halling-Sørensen et al., 1998, Heberer, 2002). In short, the rise of antibiotic consumption will increase the release of antibiotics to aquatic environment, as well as increase the number of antibiotic resistant organisms and genes, which in turn has a potentially adverse impact on human health. Of all the antibiotics present in the environment, ciprofloxacin (CIP), a quinolone antibiotic, is widely detected, sometimes at very high concentrations; up to 6.5 mg/L in lakes and 2.5 mg/L in rivers in India (Fick et al., 2009). Also, CIP is one of the antibiotics that poses a high ecological risk (risk

quotient $RQ > 1$) to the most sensitive aquatic organisms (Zhang et al., 2012). It was also demonstrated to have an influence on algal, phytoplankton and zooplankton community structure in ecosystems at a concentration of few to hundreds $\mu\text{g/L}$ (Richards et al., 2004, Wilson et al., 2003).

WWTPs are the major source for the release of antibiotics, and antibiotic resistant bacteria and genes to the environment (Michael et al., 2013, Rizzo et al., 2013). Hence, to reduce the discharge of antibiotics into the environment, as well as treat wastewater to comply with the discharge standards, or furthermore to be recycled, a more thorough treatment of wastewater is needed. The common methods for wastewater treatment employ physical, chemical and biological; however, it is widely known that biological methods are more cost-effective than physical or chemical treatment in most cases. Biological treatment can be divided into two major types; aerobic and anaerobic treatment. Microorganisms under aerobic conditions convert organic matter into carbon dioxide, new biomass, and water, whereas under anaerobic conditions microorganisms convert organic compounds into methane, carbon dioxide, ammonia, and biomass. Historically, aerobic rather than anaerobic treatment is often the treatment method of choice, however, anaerobic treatment has become more attractive recently due a variety of advantages. Anaerobic have advantages over aerobic processes in terms of less sludge production, lower energy requirements, and biogas production. Until recently anaerobic treatment was only used for high-strength wastewater with the use of anaerobic filter processes, upflow anaerobic sludge blanket (UASB) reactor, and the fluidized bed reactor. Nevertheless, the use of anaerobic treatment for low-strength wastewater has attracted considerable interest since the early 2000s when a membrane was used in conjunction with anaerobic reactors. In general, continuous stirred-tank reactors (CSTRs) operate at the same high hydraulic retention time (HRT) and solids retention time (SRT), but when coupled with a membrane, separate control of the SRT and HRT can be achieved, leading to an increase in biomass concentration and volumetric conversion rate. Thus, the anaerobic membrane bioreactor (AnMBR) can result in very effective treatment, with chemical oxygen demand (COD) removals generally greater than 90% (Hu and Stuckey, 2006, Martinez-Sosa et al., 2011, Smith et al., 2015), and in some cases, as high as 99% (Bailey et al., 1994). AnMBRs were showed to be effective at different conditions, even at short HRTs (1h) (Kunacheva et al., 2017c), psychrophilic temperatures at as low as 3 °C (Smith et al., 2015), under

different organic loading (5 to 12 kg COD/m³/d) (Win et al., 2016), and in treating pharmaceutical and chemical industries wastewater (Svojitka et al., 2017). However, there has been not been any studies carried out in the literature on the operation of an AnMBR with antibiotics in the feed. When applying an AnMBR to treat wastewater containing antibiotics, there are two important considerations, the first is whether the environmental concentration of the antibiotic will affect the performance of the reactor due to their adverse effect on the microorganisms; the second is whether the AnMBR can remove the antibiotic, and if so, by what mechanism(s).

Another concern about the AnMBR system is that despite its good performance, the effluent still needs further treatment to attain reuse or even discharge standards. If the use of fresh water from natural sources is limited in some countries, then the demands for wastewater reuse increases. There have only been a few studies on the post-treatment of AnMBR effluent (Grundestam and Hellstrom, 2007, Kim et al., 2015, Trzcinski et al., 2011, Vyrides et al., 2010); however, these were a limited number, and only looked at a small number of post-treatment processes. The limited number of post-treatment methods which have been investigated include; activated carbon, Ultrafiltration (UF) membranes (1 kDa), coagulation-flocculation, activated carbon combined with anaerobic digestion and activated carbon combined with aerobic bacteria. Activated carbon, and the combination of activated carbon /aerobic bacteria, was one of the most effective treatment methods to remove organic carbon (Trzcinski et al., 2011, Vyrides et al., 2010). However, studies investigating in detail about what compounds, especially micropollutants such as soluble microbial products (SMPs), remain or are removed from an AnMBR effluent are few and far between.

In brief, antibiotics are widely present in wastewater, and in some cases, are present in extremely high concentrations, for example CIP. The removal of antibiotics in WWTPs will help prevent the spread of antibiotics into the aquatic environment, which as a consequence will protect the ecosystem and potential adverse effects on human health. Of all the treatment processes available, the AnMBR has considerable potential for treating wastewater, and can prevent the loss of biomass inhibited by antibiotics. However, there is a lack of data available in the literature on how effective AnMBRs are in removing antibiotics. More specifically, the effect antibiotics have on anaerobic digesters, as well as on the AnMBR specifically, is poorly understood. Thus, more research is needed to better understand the removal of micropollutants and the

adaptation of microorganisms to toxicity, and to better understand biological behaviour in these reactors, which will be investigated in this thesis. In addition, further treatment of the AnMBR effluent is needed in order to attain either discharge or reuse standards. All of this information will be critical to design and operate AnMBRs more efficiently, especially under toxic conditions, and to prevent the release of potentially harmful antibiotic resistant genes into the aquatic environment.

Hence, the OVERALL AIM of this work was to explore the effect of CIP on the operation of an anaerobic batch reactor and AnMBR, and then investigate the mechanism of CIP removal and the post-treatment of AnMBR effluent by adsorption, with the main aim of removing soluble microbial products (SMPs) and CIP.

This thesis is organized as follows: in Chapter 2, a comprehensive review of the concepts of anaerobic digestion, the AnMBR, the presence of PPCPs in the aquatic environment, and post-treatment methods for anaerobic effluent. Details of an AnMBR system, the experimental procedures, and analytical methods are presented in Chapter 3. Chapters 4 and 5 showed the effect of the antibiotic CIP on anaerobic digestion in a batch study, and its effect on an AnMBR system. The effects of CIP in terms of the general parameters such as biogas production, COD removal, volatile fatty acids (VFAs) accumulation, and SMP accumulation by recently developed analytical methods, and the effect on microorganisms were investigated. This is followed by Chapter 6 that discusses the fate of CIP when removed in an AnMBR, while Chapter 7 presents the post-treatment of an AnMBR effluent by activated carbon adsorption. Finally, a concluding discussion, and the outcome of the research study and recommendations for future research are presented in Chapter 8.

CHAPTER 2

Literature review¹

Outline:

- 2.1 Antibiotics in the aquatic environment
- 2.2 AnMBR for treatment of wastewater
- 2.3. Post treatment of AnMBR effluents for recycling
- 2.4 Summary of the literature
- 2.5 Research objectives

2.1 Antibiotics in the aquatic environment

2.1.1 Antibiotics and antibiotic resistance

Antibiotics are pharmaceutical compounds that are used to treat and/or prevent bacterial infections, and the discovery and production of antibiotics is considered as one of the most important discoveries in human health. Antibiotics are classified into two categories; antibiotics that are synthesized by microorganisms, and the synthetic drugs including sulphonamides and the quinolones. Recently, semi-synthetic drugs have been developed that are a combination between synthetic and natural antibiotics.

The first antibiotic discovered was Penicillin, which was first isolated from *Penicillium notatum* in 1928 (Fleming, 1929), although at that time it could not be isolated or purified enough to use medically. In 1940 the therapeutic value of penicillin was shown (Chain et al., 1940), and after this considerable effort was expended in searching for *Penicillium* strains to produce more penicillin. After penicillin came into use, various

¹ Part of this chapter is published as Mai, D., Kunacheva, C. and Stuckey, D. (2018). A review of post-treatment technologies for anaerobic effluents for discharge and recycling of wastewater. Crit. Rev.

antibiotic compounds were discovered, including streptomycin (Schatz et al., 1944), gramicidin (Hotchkiss and Dubos, 1941), chlortetracycline, chloramphenicol, and others (Garrod and O'Grady, 1971).

In 1962, nalidixic acid, a synthetic drug, was developed, and chemical modifications of nalidixic acid produced the highly effective antibiotics belonging to the class of fluoroquinolones. They have enormous importance in treating human and animal diseases, and include CIP, enrofloxacin, ofloxacin, and norfloxacin (Mitsuhashi, 1993). After that time, only a few new antibiotic drugs were discovered and most of the new drugs were the chemical modification of existing drugs. These modified drugs enhanced the killing efficiency of pathogens, increased the broad-spectrum antibiotics, and reduced toxicity and side effects. From 1970, only one class of antibiotic, the oxazolidinones, were discovered and introduced in clinical use. Since then, the trend in antibiotic therapy has been to combine drugs of different mechanisms of action to enhance their efficiency, and to overwhelm the problem of antibiotic resistance.

Antibiotic resistance is the resistance of a microorganism to an antibiotic that occurs when bacteria change in a way to reduce or eliminate the efficacy of drugs. The mechanisms of antibiotic resistance are divided into four main groups including; modification of the antibiotic by enzyme through hydrolysis or chemical modification, alteration of the drug target, reducing the uptake by changes in membrane permeability or increasing efflux of the drug, and auxotrophia (Silva, 1996). It was evident from the beginning of the antibiotic era that bacteria can become resistant to antibiotics, and microorganisms resistant to drugs were found almost as soon as antibiotics were discovered. However, there is a strong correlation between antibiotic consumption and antibiotic resistance (Goossens et al., 2005). When penicillin started to be used extensively, the resistant strains of *Staphylococcus aureus* in hospitals were less than 1%, but that number increased to 14% in 1946, and then to 38% only one year after that, and today it is more than 90% (Greenwood, 2007); resistance to ampicillin was also found in more than 80% of the *S. aureus* strains worldwide (O'Brien, 1987). Even with the newer antibiotics such as fluoroquinolones and quinupristin/dalfopristin, resistance was still reported (Freeman, 2001, Srinivasan et al., 2002). Thus, the proper use and disposal of antibiotics is becoming very important nowadays.

2.1.2 Presence of antibiotics in the aquatic environment

Antibiotic consumption is approximated to lie between 100,000 and 200,000 tons per year globally (Wise, 2002). During their excretion (due to the lack of uptake into the human body), antibiotics can be discharged into wastewater. It is estimated that approximately 412 tonnes of antibiotics were used in healthcare in 1998 in Germany (based on excretion rate), and 305 tonnes were estimated to end up in municipal waste (Kümmerer and Henninger, 2003).

The concentration of antibiotics in hospital effluent were in the high $\mu\text{g/L}$ concentration, while they were lower in municipal wastewater. For example, for the antibiotic CIP was measured at high concentrations in pharmaceutical manufacturing and hospital WWTPs, with concentrations of 0.5-35 and 0.5-5 $\mu\text{g/L}$, respectively, whereas in municipal WWTPs, the number was 0.1-0.2 $\mu\text{g/L}$ only (Sim et al., 2011). Moreover, it was shown that more than 80% of faecal samples in healthy people contained resistant organisms (Reinthal et al., 2003). As a consequence, the resistant microorganisms and antibiotic residues will enter the sewage system during the excretion process; thus, raw sewage usually contains antibiotics and antibiotic resistant bacteria.

Due to incomplete treatment and disposal, antibiotics were found in surface waters and ground water that in many cases were at a concentration of $\mu\text{g/L}$ (Kümmerer, 2009). Many of antibiotics were detected at frequency of 100% in the river discharge to Laizhou Bay, China, and various antibiotics were found in a sewage treatment plant, surface water and ground water, as shown in Table 2.1. Generally, antibiotics were not found in drinking water except for a few rare cases where tylosin, sulfamethoxazole, macrolides and quinolones were measured at concentrations of a few ng/L (Ye et al., 2007, Zuccato et al., 2000). In addition, in one case study, the antibiotic CIP was detected at exceptional high concentrations in a lake, at 2.5-6.5 mg/L (Fick et al., 2009). Moreover, CIP was also detected in the groundwater, and up to 14 $\mu\text{g/L}$ CIP was detected in well water samples (Fick et al., 2009). The high concentration was caused by the incomplete treatment of a wastewater treatment plant in an Indian Plant, Patancheru Enviro Tech Ltd treating wastewater from 90 bulk drug manufacturers (Larsson et al., 2007), and the effluent was found to contain concentrations of up to 31 mg/L .

**Table 2.1 Examples of antibiotics measured in the aquatic environment.
Modified from (Kümmerer, 2009)**

	Antibiotic	Sewage treatment plant effluent (ng/L)	Surface water (ng/L)	Ground water/bank filtrate (ng/L)
Penicillins	Penicillin	200	3	
	Flucloxacillin		7	
	Piperacillin		48	
Macrolides	Macrolide	700	20	
	Azithromycin		3	
	Erythromycin	287-6000	15.9-1700	49
	Clarithromycin	38-328	20-260	
	Roxithromycin	68-1000	14-560	26
Quinolones	Ciprofloxacin		9-30	
	Norfloxacin		120	
	Ofloxacin	82	20	
Sulfonamides	Sulfonamide	1000	40	
	Sulfamethoxazol	370-2000	52-1900	410-470
	Sulfamethazin		220	160
	Sulfamethizol		130	
	Sulfadiazin			17
	Sulfadimidin		7	23
	Sulfadimethoxin		60	
Tetracyclines	Tetracyclin		110	
	Chlortetracyclin		690	
	Oxytetracyclin		340	
Others	Trimethoprim	38-660	24-710	
	Ronidazol			10
	Chloramphenicol	68-560	60	
	Clindamycin	110	24	
	Lincomycin		730-249	
	Spiramicin		74	
	Oleandomycin		3	
	Tylosin		3-280	

2.1.3 Effect of antibiotics on aquatic organisms/human health

The detection of PPCPs in the environment does not necessarily mean that they are causing harm immediately or directly. However, with currently limited studies, the potential toxicological and ecological impact on aquatic ecosystems has become severe (Kümmerer, 2003). If antibiotics are not eliminated during treatment processes, they will enter the environment and cause adverse effects to aquatic and terrestrial organisms. The USEPA ecological structure–activity relationship (ECOSAR) has been used to classify and evaluate four pharmaceutical classes, including antibiotics ($n = 226$) (Sanderson et al., 2004). The results predicted that 20% of the antibiotics were very toxic to algae ($0.1 \text{ mg/L} < \text{EC}_{50} < 1 \text{ mg/L}$), 16% were extremely toxic ($\text{EC}_{50} < 0.1 \text{ mg/L}$) and 44% very toxic ($\text{EC}_{50} < 1 \text{ mg/L}$) to daphnids, a planktonic species. Moreover, fish were also being affected, and almost 1/3 of the antibiotics were very toxic to them ($0.1 \text{ mg/L} < \text{EC}_{50} < 1 \text{ mg/L}$), while more than half were toxic ($\text{EC}_{50} < 10 \text{ mg/L}$). Kümmerer summarized many studies and showed that the effect of antibiotics on bacteria and microalgae was 2-3 times lower than on the higher trophic levels (Kümmerer, 2009). The same study also showed that the exposure of antibiotics to bacteria were via chronic pathways rather than acute ones. In addition, the toxicity of some antibiotics toward microorganisms, for example *Vibrio fischeri* was shown in a long-term assay (24 h), not for a short incubation period, and they affected them over a long-term assay for almost all substances (Kümmerer et al., 2004, Thomulka et al., 1993). Hence, the other consequence of long-term exposure of antibiotics to ecosystems could occur which we are not aware of. In addition, it is also noteworthy to know that different antibiotics have a common mechanism of action where the impact of a mixture will cause an additive or synergistic effect that results in a greater impact than the compounds individually (Daughton and Ternes, 1999). For example, a mixture containing the antibiotic sulfamethoxazole and carbamazepine and ibuprofen at low concentrations that can be found in the environment was tested on human and zebra-fish cells in in-vitro tests (Pomati et al., 2006, Pomati et al., 2007). The study showed that the mixture of drugs can inhibit cell proliferation, even at concentrations of ng/L. This demonstrates the potentially adverse effect of antibiotics, as well as other drugs, to ecosystems, even at low concentrations.

When calculating the risk quotients of thirteen antibiotics, CIP was shown to be one of three antibiotics that pose a high ecological risk to the most sensitive aquatic organisms,

Vibrio fischeri, *Microcystis aeruginosa* and *Synechococcus leopoliensis* (Zhang et al., 2012). In another study CIP affected *Microcystis aeruginosa* and *Lemna minor* at such low concentrations that the half maximal effective concentration (EC₅₀) toxicity values were found to be 17 and 203 µg/L, respectively (Robinson et al., 2005). Primary DNA damage showed a strong correlation with CIP concentrations, and the lowest observed effect concentration (LOEC) as indicated by the umuC test for CIP was 5.2-5.9 µg/L (Hartmann et al., 1999). In real hospital wastewater samples the genotoxicity was demonstrated to be caused mainly by fluoroquinolone antibiotics, especially CIP, although CIP was only detected at low concentrations, from 3-87 µg/L (Hartmann et al., 1998).

However, the impact of antibiotics on the environment is not just their adverse effects on the microbial communities and ecosystem, but also on how the changes affect human health through antibiotic resistance. It was shown that the bacteria isolated from bioreactors treating sewage exhibited considerable resistance to various antibiotics, including trimethoprim, erythromycin, tetracycline, CIP and ampicillin (Costanzo et al., 2005). Moreover, even at low concentrations, antibiotics were shown to induce antibiotic resistant genes in the environment (Dantas et al., 2008). Horizontal transfer of resistance genes was promoted at a level of CIP from 0.5–10 µg/L (Beaber et al., 2004). Urban WWTPs are the main source of the release of antibiotic resistant bacteria and genes into the natural environment (Rizzo et al., 2013), and the use of antibiotic contaminated water as a drinking-water source, such as in the contamination of wells in India with CIP, could be the direct route for resistant bacteria to reach humans (Fick et al., 2009). Thus, the control of antibiotic resistant bacteria from spreading into the environment is gaining attention in order to protect human and animal health.

2.1.4 Removal of antibiotic in WWTPs

The removal of antibiotics in real WWTP systems has been investigated widely, and the performance of various WWTPs using biological treatment was summarised in a recent review paper (Michael et al., 2013). In this study, the removal of antibiotics belonging to different groups was investigated, including β-Lactams, macrolides, sulfonamides, trimethoprim, quinolones, tetracyclines and other groups. However, the removal efficiency of many antibiotics varied widely, with removal efficiencies ranging from very little to nearly complete removal (Michael et al., 2013). For example, amoxicillin can be removed from 4-100%, sulfamethoxazole from < 0 to 100%.

Moreover, the review also highlighted the missing details of most studies, ie. the operational details and/or the fate of removal during wastewater treatment was not investigated. Another study showed that the elimination of antibiotics was mainly by their biodegradation, or sorption onto the sewage; hydrolysis can then happen for some compounds, while photolysis was not like to occur (Michael et al., 2013). Nevertheless, in a closed bottle test of 18 antibiotics, most of them were not readily biodegradable, with a degradation of only 4-27% (Alexy et al., 2004). In another study the authors investigated the biodegradation of 9 antibiotics under anaerobic conditions, and showed that these compounds are non-biodegradable except for benzylpenicillin which ultimately biodegraded after 40 days (Gartiser et al., 2007). A similar results were obtained under aerobic conditions, and most of the antibiotics were not readily biodegraded (Kümmerer, 2009). The removal of some fluoroquinolone antibiotics, including CIP, norfloxacin, ofloxacin, and lomefloxacin, were shown to occur mainly due to sorption which contributed from 60-100% of the total removal (Verlicchi et al., 2012). The high adsorption capacity was assumed to be due to the electrostatic and hydrophobic interactions that form flocs of microbial activity, and the electrostatic interactions between the antibiotic compounds and the sludge (Verlicchi et al., 2012).

In addition, for all the processes treating antibiotics that were investigated by Michael et al. (2013), WWTP with CAS was dominant, followed by MBRs. In general, the removal of antibiotics in MBRs was higher than in CAS (Clara et al., 2005, Radjenović et al., 2009), for example, for Sulfamethoxazole it was 18-50% removed in CAS, while it was 61-100% removed in an MBR (Radjenović et al., 2009). However, the study of antibiotic removal in anaerobic reactors is very limited. One study examined the removal of pharmaceuticals, including the antibiotic compounds macrolide, quinolone and sulphonamide in a two-stage anaerobic fluidized membrane bioreactor (AFMBR) using GAC as the carrier medium. It was shown that the removal efficiency of all antibiotic compounds including sulphonamide antibiotics, macrolides antibiotics, and cephalosporins was low, with a removal efficiency from 36-70%, whereas others such as the quinolone antibiotics, and trimethoprim had a higher removal of 81-95%. When adding one more stage to the AFMBR the removal increased significantly, resulting in an overall removal of all antibiotics of 86-100% (Dutta et al., 2014); however, the removal mechanism of this process was unknown.

2.2 AnMBR for treatment of wastewater

2.2.1 Fundamentals of anaerobic digestion

Biological processes for the treatment of wastewater can be divided into two major types; aerobic treatment, and anaerobic treatment. Historically, aerobic rather than anaerobic treatment has often been the treatment method of choice. Recently, however, anaerobic treatment has become more attractive due to its advantages in terms of low energy consumption and low sludge production. Anaerobic degradation is complex; a simple schematic is shown in Figure 2.1. In general, the breakdown of complex organic compounds involves different types of bacteria such as hydrolytic, fermentative, acetogenic and methanogenic bacteria. In the first stage the large organic compounds, for example carbohydrates, proteins, lipids, are hydrolysed into simpler compounds such as sugars, alcohols, fatty acids and amino acids, by hydrolytic bacteria. The small compounds produced from the previous stage can be transported through the cell membrane and converted into volatile fatty acids, alcohols, ketones, carbon dioxide and hydrogen by the group of acidogenic bacteria. The next stage is the activity of two groups of acetogenic bacteria; one group converts volatile fatty acids into acetates, hydrogen and carbon dioxide, while the other maintains a low level of hydrogen and formate in the presence of methanogens. Methanogenic bacteria occupy the final step, which is the conversion of intermediate compounds into simpler end products, methane and carbon dioxide. These reactions are carried out by various groups of micro-organisms.

Figure 2.1 Reaction scheme for anaerobic digestion. Adapted from McCarty and Smith (1986)

Hydrolytic Bacteria

Hydrolysis is the first reaction in the sequence of various reactions in anaerobic digestion that contribute to breaking down the larger compounds into simple and smaller solutes for further degradation. In this process, the extracellular enzymes such as cellulases, proteases and lipases will be used to hydrolyse the macromolecules into smaller compounds. In the case when large amounts of particulates are present in the feed, the degradability is reduced, and hydrolysis will become the rate-limiting step of the overall process. Under anaerobic conditions, the breakdown of these organics in these steps are carried out by hydrolytic bacteria, including *Clostridium*, *Micrococcus*, *Peptococcus* and *Staphylococcus* (Zehnder, 1988).

Anaerobic Acidogenesis

The acidogenic bacteria convert amino acids, sugars (through fermentative bacteria), fatty acids and alcohols (through anaerobic oxidative bacteria) into VFAs, alcohols,

ketones, carbon dioxide and hydrogen. They are fast growing bacteria, and have a minimum doubling time of 30 minutes, or less. The degradation of glucose, which is the most readily degradable substrate (Mosey, 1983), involves the following reactions:

The preferred pathway is dependent on the concentration of hydrogen. At low hydrogen concentrations, the first pathway through acetate is preferred, whereas, at high organic loading rates, more hydrogen is produced from the first reaction, and this triggers the second and third reactions. The formation of acetate (Eq. 2) requires two moles of hydrogen and produces one more mole of acid compared to the conversion of butyrate (Eq. 3); thus propionate formation will reduce hydrogen in the system (Mosey, 1983). In addition, the anaerobic fermentation of glucose was demonstrated to be reliant on bacterial selection through pH and substrate concentrations (McCarty and Mosey, 1991), and there is a competition between propionic and butyric producing bacteria. For example, in the presence of high concentrations of substrate and neutral pH, propionate-forming bacteria such as *Propionibacteria* and *Enterobacteriaceae* will grow quickly and produce propionate, acetate and formate from glucose. This process is not affected by the hydrogen partial pressure. In contrast, butyrate and hydrogen will be produced from butyric acid bacteria such as *Clostridia* to adapt to low-substrate concentrations and low hydrogen partial pressures. Moreover, the presence of end products of this process are strongly dependent on the methanogens. Under normal operating conditions, most substrate goes through acetate (70%), carbon dioxide and hydrogen; however, when methanogenic activity is not high enough, more high energy compounds are produced with more longer chain intermediates (C3-C7), fatty acids or ethanol and lactate (Schink, 1988).

Acetogenesis

The second main step is the oxidation of VFAs and alcohols into acetate, hydrogen and carbon dioxide based on the three reactions below (Eq. 4-6) (Mosey, 1983); this step involves acetogenic bacteria such as *Syntrophobacter wolini* and *Syntrophomonas wolfei*. The presence of this group of bacteria depends on the presence of methanogens and sulphate reducing bacteria to maintain low levels of hydrogen and formate. When dissolved hydrogen accumulates, and the partial pressure of hydrogen is greater than 10^{-4} atm, the reaction is stopped thermodynamically (Kasper and Wuhrmann, 1978). Even under low concentrations of hydrogen (10^{-6}), these bacteria still grow relatively slowly, with a minimum doubling time of 1.5-4.0 days.

Hydrogen consuming acetogenesis

The hydrogen consuming acetogenic bacteria such as *Clostridium aceticum*, prefer a mildly acidic environment to convert glucose to acetate with hydrogen consumption. This process competes with methanogens for hydrogen utilisation (Phelps and Zeikus, 1984)

Methanogenesis

In methanogenesis, methane is mainly produced from acetate or hydrogen and carbon dioxide or formate. Ten methanogenic reactions were proposed that produce methane from hydrogen, acetate, formate, methanol, carbon dioxide, monoethylamine, dimethylamine, trimethylamine, methyl mercaptans and metals (Chynoweth, 1996), and methanogenesis is usually the rate limiting step in the overall process of anaerobic

digestion. Acetate is an important intermediate metabolite in methanogenesis, and its decomposition is the rate-limiting step of the overall reaction (Mountfort and Asher, 1978). There are two pathways for methane production from acetate which have been reported to date (Petersen and Ahring, 1991, Schnürer et al., 1994).

The first pathway is direct methanogenesis by aceticlastic methanogenic archaea (Eq. 9), corresponding with the conversion of the -CH₃ and -COOH group of acetate to CH₄ and CO₂, respectively. Only members of the *Methanosarcinales* are capable of acetoclastic methanogenesis (Fournier and Gogarten, 2008, Vanwonterghem et al., 2016), with two known genera of methanogens, *Methanosarcina* and *Methanosaeta*, which can carry out this process (Hattori, 2008). They are slow growing microorganisms with a minimum doubling time of 2-3 days, and are believed to be unaffected by dissolved hydrogen concentrations (Mosey, 1983). This methanogenic pathway is the foremost pathway with the presence of either *Methanosarcinaceae* or *Methanosaetaceae* (*Pavlostathis* and *Giraldo-Gomez*, 1991, *Zinder and Koch*, 1984); whereas if *Methanosaetaceae* were not present, acetate oxidation would be dominant. It is estimated that around 70% of the methane is produced through acetate fermentation (Jeris and McCarty, 1965).

Aceticlastic cleavage

Non-aceticlastic oxidation

The second methanogenic pathway is non-aceticlastic oxidation (Eq. 10-11). This pathway comprises from 74% to 88% of the total acetate degradation (Sasaki et al., 2011), with methane production contributing from 3-89% during the course of acetate degradation <3-87% (Mulat et al., 2014); 35-89% (Sasaki et al., 2011), 80% (Hao et al., 2010) and 13.1-21.3% (Hori et al., 2011). Acetate decomposition is affected by many factors, including temperature, substrate composition, reactor type, and the organic

loading rate (Hattori, 2008). The co-metabolic pathway includes acetate-oxidizing bacteria and hydrogenotrophic methanogens, in which the syntrophic acetate-oxidizing bacteria belong to *Clostridium* spp., whereas *Methanomicrobiales* or *Methanobacteriales* are members of the hydrogenotrophic methanogens (Hattori et al., 2000, Petersen and Ahring, 1991, Schnürer et al., 1997).

2.2.2 AnMBR for the treatment of low-strength wastewaters

In the past, anaerobic reactors were constructed to treat medium- to high-strength wastewater with CODs greater than 1,000 mg/L. Low-strength wastewater (200-500 mg/L) employing anaerobic treatment had a number of problems attributed to low substrate concentration and the low growth of biomass in the reactors. However, recently it has been shown that low-strength wastewater can be treated using anaerobic reactors effectively (Lin et al., 2013).

The use of an anaerobic filter process to treat medium-strength synthetic wastewater was carried out by Young and McCarty (1969). In anaerobic filters, biomass can be formed as a granular or thick flocculent sludge, or can attach to a support material, hence, biomass will be kept inside the reactor, leading to lower suspended solids concentrations in the effluent. The process fed with an influent of 1,500-6,000 mg COD/L removed 93% COD with a hydraulic retention time (HRT) of 72h; however, removal efficiency decreased to 60% after the HRT dropped to 12 h. After that, in 1983 an UASB reactor cultivated with granular sludge was used to treat raw domestic wastewater at temperatures from 8 to 20°C, resulting in 65-85% COD removal under dry weather conditions, and 50-70% in the case of heavy rainfall (Lettinga et al., 1983). In a UASB particulates were removed by combining physical processes including adsorption, settling, entrapment and/or biological processes in the sludge bed (Ozgun et al., 2013). Sanz and Fdz-Polanco (1989) investigated the use of an anaerobic fluidized bed reactor compared with an UASB; the study showed the fluidized bed reactor gave better removal of COD, BOD₅ and TSS, as well as an easier and shorter starting up period. This finding was also supported by a study comparing the performance of four different processes: UASB, up-flow anaerobic filter, anaerobic fluidized bed, and a sludge recycling process fed with synthetic wastewater from 100 to 2,000 mg/L total organic carbon (TOC) (Kida et al., 1991). The results showed that all processes removed TOC efficiently when treating low-strength wastewater; however, the anaerobic fluidized bed reactor maintained high removals (80% of TOC removal) even when the temperature dropped

to 15°C, while other reactors performed with less efficiency. EGSB reactors are a modification of the traditional UASB that can operate at higher superficial velocities, and achieve similar or better performance than a UASB at shorter HRTs (Jeison and Chamy, 1999).

The study of AnMBRs has been popular from the early 2000's. Membranes integrated with an anaerobic reactor can keep biomass and substrate inside the reactor resulting in highly effective treatment compared with a conventional anaerobic reactor. In addition, when a CSTR is coupled with a membrane, separate control of the solids retention time (SRT) and HRT can be achieved instead of operating with the same HRT and SRT in a CSTR. This leads to an increase in the biomass concentration, and also the volumetric conversion rate. An et al. (2009) showed that effluent from a AnMBR had a lower TOC by 14.2% and 8.8% at HRTs of 10 and 5.5h, respectively, compared with a UASB supernatant. Membranes can be integrated into different types of anaerobic reactors including CSTRs, UASBs and expanded granular sludge beds (Chu et al., 2005, Wen et al., 1999). The reactor can also achieve COD removals as high as 99% (Bailey et al., 1994), and even at psychrophilic temperatures (8-15°C) about 90% COD removal was achieved (Martinez-Sosa et al., 2011). Even when the operating temperature was as low as 6 °C, more than 95% COD removal was achieved for an AnMBR treating a simulated domestic wastewater, and removal efficiencies can still be as high as 86% at 3 °C (Smith et al., 2015). Good performance of an AnMBR was also demonstrated under different reactor configurations (Harb et al., 2015), organic loadings (Win et al., 2016), and the existence of cellulose suspended solids (Watanabe et al., 2016).

Furthermore, there are some papers which have studied the removal of organic micropollutants by membrane filtration; micropollutants are present in wastewater in the range of 0.1 and 10 µg/L or even higher (Luo et al., 2014). However, some compounds such as alkylphenols, hormones and pharmaceuticals (bisoprolol) are degraded less under anaerobic conditions compared with aerobic systems (Agargues Llamas et al., 2012, Lahti and Oikari, 2011). The low removal efficiency of pharmaceuticals mainly depends on their hydrophobicity, the presence of electron withdrawing and donating groups, and nitrogen in their molecular structure (Monsalvo et al., 2014, Wijekoon et al., 2015). Hence, hydrophobic contaminants, and compounds contained electron donating groups and/or nitrogen atoms, show high removal efficiencies. In contrast, hydrophilic compounds and those containing strong electron withdrawing groups had

low removals. However, the removal of antibiotics using an AnMBR has not been examined in the literature, although antibiotics are widely found in sewage, and can cause adverse effects to microorganisms, even at low concentrations.

2.2.3 Characteristics of an AnMBR effluent

After biodegradation under anaerobic conditions, effluents still contain a variety of organic compounds, including non-biodegradable pollutants present in the feed wastewater, intermediates and end products of biodegradation processes, and soluble microbial products (SMPs) which are released during metabolism and biomass decay. Hu and Stuckey (2006) showed that membranes rejected most of the high MW soluble organics, and hence high COD removal was achieved. Size exclusion chromatography was also used by Trzcinski and Stuckey (2009) confirming that the microorganisms degraded high MW compounds almost completely, and hence they were not present in the permeate, while low-MW fulvic-like and medium MW material still appeared in the permeate of the AnMBR. Under steady state most of the effluent COD from an AnMBR were SMPs which contributed up to 95% of the effluent COD (Aquino and Stuckey, 2003, 2004, 2006). However, under stress conditions such as during nutrient starvation, intermediate volatile fatty acids (VFAs) were the main organic constituents of the effluent (Aquino and Stuckey, 2003). The molecular weight (MW) distribution of the effluent showed that SMPs have a wide range of molecular weights (<0.5 kDa up to >300 kDa) in which the majority were in the low-MW range (up to 89% w/w <1 kDa) (Aquino et al., 2006, Aquino and Stuckey, 2002, Barker et al., 1999). Some of the SMPs have been identified as humic substances, polysaccharides, proteins, nucleic acids, lipids, carbohydrates and small molecules (Barker and Stuckey, 1999, Kunacheva and Stuckey, 2014). Preliminary results on the identification of the low-MW SMPs (<580 Da) using gas chromatography mass spectrometry (GC-MS) revealed that most of compounds were long chain alkenes (C12-C24) and alkanes (C12-C16), acids and esters as well as some aromatic compounds (Aquino and Stuckey, 2002, Wu and Zhou, 2010, Zhou et al., 2009). These compounds were similar to those from an aerobic bioreactor; however, anaerobic effluent contained more types of solutes and were more complex than aerobic ones (Zhou et al., 2009). In addition, under different modes of operation of the reactor, the composition of organic compounds in the effluent were also different. For example, it was demonstrated recently that with different feed compositions the SMPs in anaerobic effluent are different in terms of size distribution, low-MW SMP

compounds and the free amino acid accumulation. More carbohydrates of the size fraction 30 k–200 kDa were found under a pH 5 shock, while higher MW protein-like compounds, 1500 kDa–0.2 μ m, were found under a pH 11 shock (Kunacheva et al., 2017b). In addition, during the transition of different HRTs from 4h to 2h and from 2h to 1h, increases in some low-MW compounds such as cyclooctasulfur, camphor, 2-methylphenol, N-butyl-benzenesulfonamide, etc. were found (Kunacheva et al., 2017c). However, the characterisation of the compounds presents in effluents, especially the SMPs, is not fully understood, although they are usually the main components of the effluent COD (Barker et al., 1999, Barker and Stuckey, 1999). In addition, they are known as recalcitrant (Barker et al., 1999), precursors of disinfection by-products (Liu and Li, 2010), and play a major role in fouling in aerobic and anaerobic membrane reactors (Jarusutthirak and Amy, 2006, Jiang et al., 2010).

Hence, the change in the characteristics of anaerobic effluents is complex and is shown in different parameters such as types/amounts of VFAs, size distribution of the organic compounds and/or SMP compounds. The formation of SMPs present in anaerobic effluents, and their changes under different operational conditions, are important as the SMPs can at times contribute substantially to the effluent COD concentration.

2.3 Post treatment of AnMBR effluents for recycling

As the use of fresh water from natural water sources becomes stressed due to population growth, reducing freshwater demands becomes imperative through the reuse of wastewater. The reused water can be applied for the irrigation of; agriculture, golf courses or residences, industry, environments such as natural systems restoration, groundwater recharge, or other non-potable uses. Technically, anaerobic effluent from a UASB, EGSB and AnMBR have quite high qualities, especially effluent from an AnMBR which has a quality high enough for discharge standards in terms of organic matter measured by COD. However, there are various other types of organic compounds such as non-biodegradable pollutants, SMP compounds or the untreated PPCPs present in the effluent that require further treatment to prevent possible harm to the ecosystem or human health. Post-treatment for recycling purposes aims to treat high quality anaerobic effluent, especially AnMBR effluent, to a higher level, where the removal of the last traces of COD, as well as specific compounds occur. The possible processes discussed below include, membranes, advanced oxidation processes, and adsorption,

which have been used or have the potential to be used for the post-treatment of anaerobic effluents for reuse purposes.

2.3.1 Membranes

Membranes can be integrated with anaerobic reactors to improve the performance of the overall process, and microfiltration (MF) or UF membranes are normally coupled with an anaerobic CSTR or UASB to form an AnMBR. The use of membranes such as forward osmosis (FO), reverse osmosis (RO) and nanofiltration (NF) for the post-treatment of AnMBR is aimed at producing a high-quality wastewater that can be reused. FO is the transport of water across a semi-permeable membrane similar to natural osmosis, and coupling FO membranes with an anaerobic bioreactor provided a higher quality effluent compared to a conventional AnMBR, especially in terms of nitrogen and phosphorus removal (Chen et al., 2014, Ding et al., 2014). In spite of the excellent performance of a FO-AnMBR flowsheet which produced durable, reliable and high quality water (Lutchmiah et al., 2014), it should be noted that salinity in the reactor increased due to salt rejection by the FO membrane. At low concentrations of salt, anaerobic bacteria did not suffer any inhibition or toxic effects (Chen et al., 2014), however, at higher salinities it had a negative impact on methanogenic growth (Tang and Ng, 2014) and caused a shift in the methanogenic community and pathways (Wu et al., 2017). Other studies indicated FO could reject particles, pathogens and emerging contaminants in wastewater (Lutchmiah et al., 2014).

Dense NF membranes have the potential to reject most multivalent ions, and RO membranes even reject monovalent ions and all multivalent ions (Visvanathan and Roy, 1997). Nevertheless, for many applications the very high energy demand of RO membranes is a major drawback. Although NF permeates still contain monovalent ions, unlike RO their high quality and the lower pressures required (and lower energy consumption) make NF attractive for water reuse. In spite of this, there have been no applications of NF to anaerobic effluent post treatment. However, RO has been used once for the post-treatment of an AnMBR treating domestic wastewater, and enhanced organic carbon removal significantly from 92% to 99% TOC for the total system (Grundestam and Hellstrom, 2007).

The role of membranes in the removal of endocrine disruptors, pharmaceuticals and personal care products has also been studied (Snyder et al., 2007), and it was shown that MF and UF membranes were ineffectual in removing nearly all organic contaminants. However, nanofiltration used after an AnMBR resulted in consistently high rejection of all 15 organic micro-pollutants tested, with an average of 87% (80-92%) rejection, and this resulted in a very high removal of organic micro-pollutants in the overall system (Wei et al., 2016). In addition, most of the compounds investigated were removed by RO membranes to below their detection limit, although some contaminants were still detectable at trace levels (Snyder et al., 2007). These results agree with a study showing that hydrophilic organics with low MWs could pass through RO membranes (Hu et al., 2003). Hence, RO, FO and NF are effective processes for treating AnMBR effluent, although some micro-pollutants were not completely removed. However, the cost effectiveness and issues of membrane fouling are drawbacks of these processes (Goosen et al., 2005).

2.3.2 Advanced oxidation

Advanced oxidation is the oxidation of organic and inorganic components by reactive intermediates such as hydroxyl radicals or sulfate radicals, although the latter has rarely been used, it has become popular recently (Liu et al., 2014). They could become a promising technology due to their higher quantum yield that induces a higher photolysis rate and lower scavenging effect (two orders of magnitude lower), corresponding with a higher yield than that of hydroxyl radicals from H_2O_2 (Pari et al., 2017). Few applications for anaerobic effluent have been documented in the literature, as shown in Table 2.4, and no application in the post-treatment of AnMBR effluent has been investigated so far. In general, advanced oxidation processes can degrade the most recalcitrant molecules such as pesticides, chlorinated compounds, phenolics and cyanides from a few thousand mg/L to less than 1 mg/L (Chong et al., 2012a). In addition, advanced oxidation can inactivate pathogens, however, they are rarely used because of the short half-life time of these radicals (Cho et al., 2005, Ikai et al., 2010). In general, advanced oxidation processes can be divided into two categories: chemical oxidation such as ozone, H_2O_2 , H_2O_2 /ultraviolet (UV) light, Fenton and photo-Fenton; and, oxidation without the addition of chemicals including UV, electrochemical, solar photocatalytic using TiO_2 , ultrasound and wet air. Hydrogen peroxide is also a strong oxidant which can result in up to 99% COD removal for the post-treatment of UASB effluent treating municipal wastewater (Bhatti et al.,

2014). Moreover, wasted H_2O_2 collected after its use from an industrial process could also be used so that the cost of treatment was reduced by approximately 50% (Bhatti et al., 2014). However, the degradation rate for treating complex and toxic wastewater is low when applying hydrogen peroxide alone. For anaerobic effluent from slaughterhouse, and textile dye baths, the photochemical reaction by combining UV light with H_2O_2 or O_3 will increase the rate of hydroxyl radical production, leading to an increase in removal efficiency (Cao and Mehrvar, 2011, Muhammad et al., 2008). In Fenton oxidation, hydrogen peroxide in acidic media decomposes into hydroxyl radicals in the presence of metal catalysts, Fe(II) or Fe(III) salts, whereas the photo-Fenton oxidation employs UV irradiation to enhance the reaction rate of hydroxyl radical production. The photo-Fenton process resulted in high performance for a UASB treating poultry manure wastewater, and removed up to 95% (overall 99%) COD (Yetilmezsoy and Sakar, 2008). However, when applied to anaerobic digestion treating reactive Cibacron Red FN-R, the process only achieved 59% DOC removal, and removal was not based on oxidation but iron coagulation (García-Montaña et al., 2008).

Thus, advanced oxidation is a promising process for the post-treatment of anaerobic effluent, as well as other wastewaters. However, there are three main problems which need to be investigated before the technique is applied for the treatment of an AnMBR effluent. The first is whether this process achieves good performance when treating an AnMBR effluent, and by what mechanism. This is important as there has been no application of this technology for AnMBR effluent so far, although there are a few studies applied to UASB and other anaerobic effluents. The second is the cost effectiveness of this process compared with others, including adsorption by activated carbon, which is widely use, and will be discussed below. Another concern is the potential of producing by-products during the oxidation of organic compounds that have even higher toxicities than the original compounds. Therefore, the use of this process to treat AnMBR effluent is still questionable, although it could be a promising method.

Table 2.2 Advanced oxidation methods for treating anaerobic effluent.

Type of post treatment	Type of wastewater	Main treatment	COD removal (%)	Overall COD removal (%)	Reference
Solar photocatalytic oxidation (TiO ₂)	Dairy wastewater	UASB	62	95	(Banu et al., 2008)
Ozonation	Industrial effluent	UASB	92	-	(Yasar et al., 2006)
Ozonation	Cellulose pulp bleaching	HAISR ^c	51	-	(Pires and Momenti, 2009)
UV	Distillery wastewater	AUFBR ^d	10	85	(Apollo et al., 2013)
UV	Industrial effluent	UASB	57	-	(Yasar et al., 2006)
H ₂ O ₂	Municipal wastewater	UASB	99	99.9	(Bhatti et al., 2014)
H ₂ O ₂ /UV	Industrial effluent	UASB	82	-	(Yasar et al., 2006)
Photo-Fenton	Dye wastewater	Anaerobic batch reactor	59 ^a	-	(García-Montaña et al., 2008)
Photon-Fenton	Industrial effluent	UASB	87	-	(Yasar et al., 2006)
Photon-Fenton	Synthetic textile effluent	anaerobic reactors	43-100	-	(Punzi et al., 2015)
Fenton	Industrial effluent	UASB	60	-	(Yasar et al., 2006)
Fenton	Poultry manure wastewater	UASB	89-95	99	(Yetilmezsoy and Sakar, 2008)
Electrochemical	Municipal wastewater	EGSB	33	87	(Dai et al., 2011)
Electrochemical	Simulated wastewater ^e	UASB	67-82	-	(Buzzini et al., 2007)

Note: ^a DOC removal; ^b TOC removal; ^c horizontal anaerobic immobilized sludge bed;

^d anaerobic up-flow fixed bed reactor; ^e simulated wastewater from an unbleached Kraft pulp mil

2.3.3 Adsorption

Adsorption is an effective treatment process for recalcitrant pollutants which are hard to remove by biological processes. Various adsorbents have been investigated, including activated carbon, polymer resins, nanosorbents such as carbon nanotubes, graphene and various types of low-cost adsorbent. Amongst all these alternatives, activated carbon is the most popular and widely used; the application of other adsorbents is limited because of either their high cost, or low adsorption capacity, or both. For the post-treatment of AnMBR effluents, adsorption by powdered activated carbon (PAC) was investigated as one of the most efficient treatment methods for removing traces of organic carbon (Trzcinski et al., 2011, Vyrides et al., 2010). PAC and granular activated carbon (GAC) had the highest COD removals of 84% and 80%, respectively, surpassing UF membranes (1 kDa), coagulation-flocculation and polymeric adsorbents which had COD removals ranging from 80% (GAC) to 32% (XAD4) (Trzcinski et al., 2011). Comparing PAC with other biological methods such as aerobic, anaerobic or combining these types of biomass with PAC, Vyrides et al. (2010) obtained similar results that PAC alone removed 80% of the dissolved organic carbon (DOC). The adsorption ability of activated carbon was high due to its porous structure which possesses a large surface area (500-1,500 m²/g), with numerous internal pores and functional surface groups. Thanks to this property, activated carbon can adsorb various solutes, including organics such as phenol and phenolic compounds (Dąbrowski et al., 2005), volatile organics (Chiang et al., 2002), pharmaceuticals (Rakić et al., 2015) and metal ions like lead, copper, zinc, chromium and cyanide (Monser and Adhoum, 2002).

Nanosorbents for water purification are known to have exceptional adsorption capacities. Carbon nanotubes showed a much higher capacity than GAC and other carbon-based adsorbents in removing aromatic organic compounds and natural organic matter due to their large surface area and surface chemistry (Amin et al., 2014). These sorbents have a similar bonding structure to activated carbon but have a higher surface area/volume due to their nanoscale. The adsorption mechanism is similar to activated carbon also, that is physical adsorption and interactions with functional groups on the surface. However, activated carbon has a limited interaction with compounds as diffusion into the porous structure is required; whereas carbon nanotubes or graphene readily interacts, so no diffusion is required. Despite this, the current cost of fabricating these nanomaterials is a big challenge to enhance their widespread use; this may also be

a reason why these materials have not been used to treat anaerobic effluent. However, when their cost reduces, these materials are worth trying in post-treatment, and could become a promising material in the future for effluent purification.

Polymer resins were considered as effective sorbents, and many have been investigated and are commercially available. Various ion exchange resins have been shown to be effective for removing natural organic matter (Bolto et al., 2004), and they are easily regenerated using acid or alkaline solution; one study showed that they are more cost effective than activated carbon when site regeneration is used (Heijman et al., 1999). However, they are not as effective as activated carbon for natural organic matter removal, while the fraction of non-removable organics by ion exchange (40%) is higher than activated carbon (<10%) (Fettig, 1999). For the post-treatment of an AnMBR effluent, 2 types of polymeric adsorbents, XAD7HP and XAD4, also demonstrated a lower capacity than activated carbon (Trzcinski et al., 2011). Besides the use of these adsorbents, various other low-cost adsorbents have been generated via carbonization and an activation process. These waste materials include household waste (fruit wastes, coconut shell), agriculture (rice husk) or industrial waste (sugar industry wastes, fly ash) (Ali et al., 2012). The use of these adsorbents is not only beneficial for water treatment, but also enhances the efficient management of solid wastes. Nevertheless, these adsorbents are effective in removing pollutants at high concentrations (mg/L), but not for trace pollutants in the $\mu\text{g/L}$ range. In addition, disposing of the saturated adsorbents is another concern for the success of these adsorbents in the future.

Besides the effectiveness and cost of adsorbents, another concern is the characterization of the organic compounds adsorbed, especially the identification of specific organic compounds removed by these adsorbents, which is limited and not fully understood yet. As adsorption is widely used for drinking water (and possibly recycling water), the removed/remaining compounds after adsorption become important, as well as overall performance such as COD, TOC removal. Some efforts have been made to characterize the organic compounds in anaerobic effluent and their removal by adsorption, especially by activated carbon. It was demonstrated that low MW compounds ($\text{MW} < 1 \text{ kDa}$) were the dominant fraction in effluents from an anaerobic reactor (Aquino et al., 2006, Aquino and Stuckey, 2002, Barker et al., 1999), and contained up to 89% by mass (Barker et al., 1999), but were more difficult to adsorb than high MW ($\text{MW} > 1 \text{ kDa}$)

compounds (Barker et al., 1999, Vyrides et al., 2010). Treatment by GAC showed that the adsorption of low MW compounds was limited, compared with high MW compounds, and that only 67% COD removal was achieved for compounds of <1 kDa, whereas 79-85% of the COD of higher MW solutes was removed (Trzcinski et al., 2011). For PAC adsorption, Vyrides et al. (2010) also showed that only 70% was removed of the <1 kDa fraction, while for the >1 kDa fraction more than 90% was removed, and for the fraction greater than 50 kDa, and from 10-30 kDa, showed 100% removal. In addition, the study revealed that high MW solutes adsorbing onto PAC during the first 15 mins can block a significant fraction of the pores, resulting in slower adsorption rates of lower MW compounds. The adsorption capacity was shown to depend on the size of AC, with small activated carbon particles (≤ 0.25 mm) adsorbing better (98% removal) than large activated carbon (≤ 0.75 mm) particles (50% removal) (Vyrides et al., 2010). Different compounds also behaved differently with different particle sizes; for example protein removal was significantly affected by particle size, while if no or little protein was present, carbohydrate removal efficiency did not depend significantly on particle size (Dizge and Tansel, 2011).

With the adsorption of specific compounds, PAC showed the ability to adsorb volatile fatty acids (VFAs) formed during anaerobic conditions, and acetate and butyrate were adsorbed at a relatively higher capacity compared to propionate (Baeta et al., 2013). Recalcitrant compounds which were commonly found in anaerobic effluent, such as bisphenol A and bis(2-ethylhexyl)phthalate, had removals of 85 and 75%, respectively, by PAC addition at 2 g/L in an AnMBR (Trzcinski and Stuckey, 2010). The study also observed that other compounds with very high hydrophobicity, such as phenanthrenecarboxylic acids and phenanthrenecarboxylic acid methyl esters, were removed at very high efficiencies of 88 and 100%, respectively. Similarly, with endocrine disruptors, and pharmaceuticals and personal care products, GAC was very effective, and the hydrophobic compounds were removed more effectively than hydrophilic ones (Nguyen et al., 2012, Snyder et al., 2007). The feasibility of using GAC for the post-treatment of MBR effluents showed the significant removal of persistent trace organics, e.g. fenoprop, ketoprofen, naproxen, diclofenac and carbamazepine, and achieved 98% or more for the overall MBR-GAC system, compared with below 40% for the MBR only (Nguyen et al., 2012). In an AnMBR system, micropollutants such as m-aminophenylacetyle, cyclohexane 1,2,4 trimethyl and cholestan 3-one, which could

be cell lysis products or material from the cell wall that is released under extreme conditions, were eliminated by PAC treatment of the effluent (Vyrides et al., 2010).

2.4 Summary of the literature review

The main conclusions from the literature review were as follows:

- Worldwide antibiotic consumption is increasing, as well as their discharge to sewerage systems. Due to their incomplete removal in WWTPs, antibiotics are discharged to various aquatic environments at concentrations of ng/L to µg/L. The presence of antibiotics in the aquatic environment may have potentially adverse effects on the ecosystem and human health through either their toxicity, or the spread of antibiotic resistant genes. This is especially true for CIP, a commonly used antibiotic, which is sometimes present in the aquatic environment at extreme high concentrations of up to mg/L. In addition, CIP was one antibiotic that showed a high impact on aquatic organisms, and its EC₅₀ toxicity values for *Microcystis aeruginosa* was as low as 17 µg/L (Robinson et al., 2005). CIP was shown to be the main factor causing genotoxicity in the real hospital sewage, although the concentrations detected were lower than 100 µg/L.
- WWTPs are the major reason for the spread of antibiotics into the aquatic environment. The removal of antibiotics in various biological treatment processes was investigated in conventional activated sludge processes, as well as in several MBR processes. The removal efficiency of antibiotics was different with different types of compounds, and a variety of processes had removal efficiencies ranging from no elimination to 100%. The mechanism of removal was not usually investigated, but where it was the experiments showed that sorption was the main mechanism generally, and most of the antibiotics were not readily biodegraded.
- Various types of anaerobic reactors have been developed and used over the last 50 years for treating a variety of wastewaters, in which anaerobic reactors have been shown to have advantages in terms of biogas production, and lower sludge production. Recently, the anaerobic membrane bioreactor (AnMBR) has become increasingly popular due to its complete biomass retention, high COD removal efficiencies, and a high-quality effluent. The AnMBR has proved to be effective operated under different conditions, including low temperature, short HRT, high organic loading, and in the presence of cellulose suspended solids. However, the

operation of AnMBR with a high level of antibiotics in the feed has not been investigated.

- Although AnMBR effluent is of a high quality, the characterisation of the organic compounds in it is still limited, and considerable efforts to analyse these solutes are still being carried out. SMPs compounds in the effluent are important as they are the main component of effluent COD in most cases, and contribute significantly to membrane fouling, as well as being precursors for disinfection by products. Recent studies have shown a change in SMP composition with different feed compositions, pH shocks or short HRT. However, the production of SMPs when exposed to CIP in anaerobic digestion as well as in anaerobic reactors is still unknown.
- The post-treatment of AnMBR effluent is required to reuse water which is necessary nowadays to reduce the demand for freshwater. Adsorption, membranes and advanced oxidation processes are potential processes for the post-treatment of AnMBR effluent. Membranes have advantages due to their high performance, although their limitations are high cost and fouling issues. Advanced oxidation processes have shown their potential for anaerobic effluents, although no investigation on treating AnMBR effluents have been carried out. In addition, their cost, effectiveness and the potential production of by-products are potential limitations of this technique. Activated carbon adsorption was shown to be one of the most effective processes to treat AnMBR effluent. However, to attain reuse standards, in addition to their effectiveness and cost of the treatment process, one concern is the characterization and identification of specific organic compounds that are removed or not, which is limited and not fully understood yet.

2.5 Research objectives

Based on the literature review above, research was carried out with the following specific objectives:

1. To determine the inhibition concentration of a model antibiotic, CIP, that is often present at low concentrations in wastewaters, and to investigate its effect on anaerobic organisms.
2. To assess the effect of the antibiotic CIP on anaerobic digestion performance, and how it influences the operation of an AnMBR

3. To determine the fate of the antibiotic CIP when it is removed in an AnMBR, and assess whether biodegradation/transformation, adsorption, or other mechanisms are responsible for its removal.
4. To measure the amount and composition of low-MW SMPs in anaerobic digestion and in an AnMBR when exposed to CIP.
5. To investigate the use of adsorption by activated carbons for the post-treatment of AnMBR effluent and identify the specific compounds/fractions which are removed or remain after adsorption.

CHAPTER 3

Materials and Methods

Outline:

- 3.1 Reactor design
- 3.2 Operation condition for AnMBR
- 3.3 Batch experiments
- 3.4 Adsorption material
- 3.5 Analytical techniques

3.1 Reactor design

The AnMBR was designed by Hu and Stuckey (2006) and used in various other studies (Kunacheva et al., 2017c, Trzcinski et al., 2011, Xiao et al., 2015). The design of the reactor used is shown in Figure 3.1, and the whole system can be divided into five major sections; the main reactor, liquid level control system, biogas recycling system, biogas collecting system and process control unit. The main body of the reactor is made of clear polymethyl methacrylate (Plexiglas®) with a total working volume of 3.2 L. Inside the reactor there is a baffle which helps guides the flow up pass the membrane in an upward direction to the surface for gas separation, and then down to complete the cycle. The top of the reactor consists of a number of inlets and outlets, while the bottom section contains a long stainless gas diffuser tube. A welded flat sheet membrane unit (polyethylene) was used in the reactor with a total surface area of 0.116 m² (size of 222 mm × 315 mm × 6 mm), and an average pore size of 0.2 μm (maximum 0.4 μm), and was heat welded onto a support plate made of acrylonitrile butadiene styrene.

A liquid level control system was set up to keep the liquid level inside the reactor constant and avoid any overflows caused by an imbalance between the influent and effluent flows, and maintain a stable pressure in the headspace of the AnMBR. A control unit and a sensor with three electrodes were used to control the operation of the peristaltic pump by detecting the liquid level position.

The biogas recycling system collects gas from the headspace of the AnMBR and pumps it via a vacuum pump to the stainless-steel tube diffuser; the diffuser has 4 large holes located in the bottom part of the tube facing downwards. The biogas is re-circulated and generates coarse bubbles which coalesce to form slug bubbles which shear the fouling layer to minimize fouling on the membrane (by cleaning the membrane) and provide good mixing for the reactor. The sparging rate was controlled by a gas flow-meter at 5 L/min.

The biogas produced was collected using a plastic bag connected to the reactor.

Figure 3.1 Schematic diagram of the AnMBR system (Kunacheva et al., 2017c).

3.2 Operation conditions for the AnMBR

The reactor was in a water bath to maintain the temperature at $35 \pm 1^\circ\text{C}$. The sludge inoculum was obtained from an anaerobic digester in Ulu Pandan water reclamation plant in Singapore, and the total suspended solid (TSS) in the reactor was 6000 mg/L at the beginning of the experiment. Oxidation-reduction potential (ORP), transmembrane pressure (TMP) and pH were monitored and recorded online continuously. ORP, pH and temperature were measured using a pH/ORP probe (Mettler Toledo, M300), while TMP was monitored by a digital pressure gauge (Ashcroft). The hydraulic retention time (HRT) was 6h except during the initial start-up period when it was 12h, and the solids retention time (SRT) was 300 days. The signal was transmitted to the personal computer using a pressure transmitter (PMP 1400, RS components) and a turbine type flow meter (Model 101-3). The pH was maintained between 6.8-7.2 using 1M NaHCO_3 and 1M HCl .

The feed for the AnMBR was prepared by diluting the concentrated feed of 40,000 mg COD/L (Table 3.1) with tap water to obtain around 500 mg COD/L. The feed composition included meat extract and peptone as a carbon and nitrogen source, respectively, with the further addition of NaHCO_3 , K_2HPO_4 and trace metals (CoCl_2 , FeCl_2 , MnCl_2 , Na_2MoO_4 , NiCl_2).

Table 3.1 Feed composition (for 40 gCOD/L)

Carbon source and macro-nutrients (g/L)		Metal nutrients (g/L)	
Glucose	26.70	$\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$	0.157
Peptone	7.99	$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.0238
Meat extract	2.68	$\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$	0.0045
K_2HPO_4	0.80	$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	0.0075
NaHCO_3	65	$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.00375

The reactors were seeded with sludge from municipal wastewater treatment plant digesters (Ulu Pandan Water Reclamation Plant, Singapore). The temperature of the reactor was maintained at $35 \pm 1^\circ\text{C}$ by operating it in a water bath. The mixed liquor volatile suspended solids (MLVSS) in the reactor were 6 g/L at the start of the experiment, and the solids retention time (SRT) was kept at 300 days.

The AnMBR was operated for 277 days, and the overall operation can be divided into 5 phases, with operational parameters listed in Table 3.2. When starting up, the HRT was set at 12 h, and then subsequently decreased to 6 h after 44 days and remained at this value until the end of the experiment. When “steady state” was achieved after 88 days from the start-up of the reactor, CIP was added to the feed at a concentration of 0.5 ± 0.1 mg/L. The reactor was operated under this condition for 41 days before the CIP concentration was increased. At first, CIP was increased to 1.5 ± 0.1 mg/L for 11 days then to 4.7 ± 0.7 mg/L for 93 days. The short period of adding CIP in period IV was aimed to prevent any possible shock to the reactor before the high concentration of CIP was added. The period V was longer (double) compared with other periods to see if any recovery of the reactor occurred after its exposure to high concentrations of CIP. In the final stage, no CIP was added to the reactor to evaluate the recovery of the reactor after antibiotic exposure.

Table 3.2 Operational condition of AnMBR during time (day)

Phase	Operating time (days)	HRT (h)	CIP in influent (mg/L)	COD feed (mg COD/L)
Phase I	44	12	0	485 ± 22
Phase II	44	6	0	487 ± 31
Phase III	41	6	0.5 ± 0.1	490 ± 28
Phase IV	11	6	1.5 ± 0.1	512 ± 21
Phase V	93	6	4.7 ± 0.7	490 ± 22
Phase VI	45	6	0	485 ± 20

3.3 Batch experiments

3.3.1 Batch experiment for anaerobic digestion

The batch experiments for anaerobic digestion were conducted in 160 ml serum bottles, and contained substrate, anaerobic sludge, and the antibiotic CIP. Anaerobic digestion sludge was taken from two laboratory AnMBRs fed synthetic wastewater, with the configuration as described in Section 3.1. The reactors were seeded with sludge from municipal WWTP digesters (Ulu Pandan Water Reclamation Plant, Singapore). The hydraulic retention time (HRT) was 12 h initially during start-up, but was then reduced

to 6 h on day 42, and remained at 6 h after that. On day 65 at steady state, with a COD removal > 95%, sludge was taken as the seed for the batch tests.

The sludge was mixed with autoclaved synthetic municipal wastewater (AnMBR feed – Table 3.1), in a serum bottle to result in a 2 g COD/L inoculation. Ciprofloxacin hydrochloride (CIP-HCl) (Sigma-Aldrich) was spiked into the serum bottles to give concentrations of 0, 0.05; 0.5; 2.5; 5; 50 mg/L (denoted as Control, CIP0.05, CIP 0.5, CIP2.5, CIP5, and CIP50, respectively), which are environmentally relevant concentrations (except for the highest concentration); the highest concentration was around twice the highest found in the environment of 31 mg CIP/L (Larsson et al., 2007). A blank was also set up using the same sludge and synthetic feed without the carbon sources (glucose, peptone and meat extract). The serum bottles were purged with N₂ for 1 min to remove all the oxygen, and then sealed with a rubber stopper immediately. Triplicate experiments were carried out in an incubating shaker at 150 rpm and 35 °C, and the gas volume and composition measured frequently. 10 mL of anaerobic sludge was taken for COD, VFA, and CIP analysis on day 0, 12, 24 and 36, and molecular analyses of the microbial communities on day 12. After 45 days, the supernatant and biomass were centrifuged and analyzed for volatile organic carbon and CIP adsorption onto biomass, respectively.

3.3.2 Kinetic experiment for the biosorption of CIP onto AnMBR sludges

The biosorption properties of CIP onto AnMBR sludges were investigated by using batch experiments. Prior to this experiment, a comparison between the biosorption of living biomass and dead biomass (ultrasonicated biomass) was conducted, and it was found that there was no statistical difference between them. In addition, a mass balance on CIP showed that >95% to 100% of the CIP added was extracted from the biomass after the biosorption test. This confirmed that under the experiment conditions used, only biosorption occurred, and no biodegradation. To obtain adsorption kinetic data, sludge from an AnMBR (without CIP addition) was taken, centrifuged and washed with DI water once before the test. The sludge was added to media which was similar to the feed composition excluding the carbon source (sugar, peptone and meat extract), and added with a CIP concentration of 5 mg/L. The samples were taken in triplicate, purged, sealed and stirred from 1 to 300 mins at 35 °C. Samples for analysis were then taken over time and centrifuged, washed once with DI water, and stored at -80 °C for analysis.

The kinetics of adsorption were described using a Pseudo-first order and Pseudo-second order kinetic model (Eq. 1, 2).

$$\frac{dq}{dt} = k_1 (q_e - q_t) \quad (\text{Eq. 1})$$

$$\frac{dq}{dt} = k_2 (q_e - q_t)^2 \quad (\text{Eq. 2})$$

$$q_e = \frac{(C_o - C_t)V}{W} \quad (\text{Eq. 3})$$

Where q_t (mg/g) is the adsorption capacity at time t (min); q_e (mg/g) is the adsorption capacity at equilibrium; and k_1 (1/min) and k_2 (g/mg/min) are the rate constants for the pseudo-first and second-order equations, respectively.

3.3.3 Isotherm experiment for biosorption of CIP onto AnMBR sludges

The isotherm experiments were carried out in a similar way to the kinetic experiments. However, in the isotherm experiment the sludge was added to a CIP solution in the media (5 mg/L) by varying the volume of solution (ranging from 7.5 to 45 mL), and then it was mixed for 24 h to ensure equilibrium. The samples were treated under the same conditions as with the kinetic experiment samples, including centrifuging, washing, storage and extraction.

The data from the isotherm experiments was fitted using either the Langmuir (Eq. 3) or Freundlich equations (Eq. 4);

$$\frac{1}{q_e} = \frac{1}{q_m} + \frac{1}{b q_m} \times \frac{1}{C_e} \quad (\text{Eq. 3})$$

$$\log q_e = \log K_F + \frac{1}{n} \times \log C_e \quad (\text{Eq. 4})$$

$$K_d = \frac{C_s}{C_e} = K_F C_e^{1/n} / C_e \quad (\text{Eq. 5})$$

Where C_e , q_m , and b are the concentrations of adsorbate in solution at equilibrium (mg/L), maximum capacity of the adsorbent for adsorbate (mg/g), and the constant for the apparent energy of adsorption (L/mg), respectively. K_f is the Freundlich constant (mg/g) and n is the Freundlich exponent constant. The K_d , sorption distribution coefficient is calculated based on Eq. 5.

3.3.4 Batch experiment for adsorption of activated carbon in AnMBR effluent

AnMBR effluent (1L) and activated carbon (1g) were added to a 1000 mL glass bottle for the adsorption experiment, and the mixed sample was stirred at 500 rpm at room temperature (25 °C) for 24h. After stirring, the samples were settled for about 10 mins, and then filtered through 0.45 µm filters to separate the liquid and solid fractions. All samples were then stored at a temperature of 4 °C, and the analyses were carried out within 10 days.

A kinetic study of one activated carbon (SA2) was also carried out to see how fast adsorption of the effluent solutes occurred. The experiments were conducted in a 100 mL glass bottle containing 100 mL of AnMBR supernatant taken during period II, with the addition of activated carbon to obtain 2 g activated carbon /L. The kinetics were modelled using a Pseudo-first order (Eq. 1) and Pseudo-second order (Eq. 2) kinetic model (part 3.3.2).

3.4 Adsorption material

Six types of activated carbon were assessed, including two GACs: NRS, FD and four PACs: SAE2, SAES, HYD, WP. They were all obtained from the Cabot and Calgon Carbon companies, and their properties as listed in Table 3.3. The characterisation of the activated carbons is described in section 3.5.6.

Table 3.3 Characteristics of activated carbons tested.

Carbon types	Denotation	B.E.T surface area (m ² /g)	Pore volume (cc/g)	Pore radius (Å)	Particle size
Filtrisorb 300 D	FD	884	0.04	16.33	d ₈₅ = 2.36 mm
NRS EA 0.5-1.5	NRS	768	0.18	18.13	d ₉₈ = 0.5 mm
Hydrosorb C	HYD	370	0.28	20.20	d ₉₉ = 150 µm
SAE2	SA2	619	0.26	18.19	d ₉₅ = 150 µm
WP-AO	WP	761	0.10	18.14	d ₈₀ = 75 µm
SAE Super	SAS	1021	0.33	18.00	d ₉₇ = 150 µm

3.5 Analytical techniques

3.5.1 pH

pH was measured with a calibrated pH meter (Jenway Model 3020), and the values obtained were accurate to within ± 0.2 .

3.5.2 Chemical Oxygen Demand (COD)

COD was measured based on the 'closed reflux colorimetric method' in section 5220-D of the APHA (1992). Sulphuric acid reagent and digestion solution were prepared as follows according to the following instructions. To prepare the digestion solution 10.216 g of dried $K_2Cr_2O_7$ for high range (100-1000 mg COD/L) and 1.022 g for low range (5-100 mg COD/L) and concentrated H_2SO_4 (167 ml) and $HgSO_4$ (33.3 g) were added to distilled water (500 ml) then mixed, cooled to room temperature and diluted to 1 L. The sulphuric acid reagent was prepared by adding Ag_2SO_4 (5.5g) to concentrated H_2SO_4 (1kg). The COD protocol consisted of adding the following; 2.5 ml sample, 1.5 ml digestion solution and 3.5 ml sulphuric acid reagent to Hach reflux tubes. The tubes were then sealed tightly, mixed properly and refluxed at 150 °C for 2 hours in a Hach COD reflux reactor (Model 45600). After this the samples were cooled and then analyzed using an UV-VIS Spectrophotometer (Shimadzu UV-2600) at wavelengths of 420 and 600 nm for low range and high range COD, respectively. The COD standard solution was prepared using potassium hydrogen phthalate (KHP) solution with a range of 5-100 mgCOD/L for low-range COD, and 100-1000 mgCOD/L for high range COD. The coefficient of variance (COV) for ten identical samples was within $\pm 5\%$, and the detection limit was 5 mg/L.

3.5.3 Volatile Fatty Acids (VFAs)

VFAs (C1-C5) were determined using a high-performance liquid chromatography (HPLC-Shimadzu HPLC SPD-20AD) system equipped with a UV diode array detector (DAD SPDM20A) fitted with an Aminex HPX-87H ion exclusion column (300 mm x 7.8 mm). The operational parameters were as follows: sample injection volume was 50 μ l, temperature was at 55 °C, mobile phase was 0.005M H_2SO_4 and flow rate was 0.8 mL/min, and the analysis time was 25 min. The VFA standards were a mixture of 7 VFAs, including 5 straight chain fatty acids from C1-C5, iso-butyric acid and iso-valeric acid. The COV for five samples was within $\pm 6\%$.

3.5.4 Total and volatile suspended solids (TSS/VSS)

The TSS/VSS measurements followed section 2540-B and 2540-E of Standard Methods (APHA, 1999). For TSS measurements, 3 ml of sludge was filtered, and the supernatant discarded, and then the sludge was dried in a pre-weighed aluminium tray at 105°C until the weight stabilised (~ 24h). The dried sample was left in a desiccator to cool, and the weight was then recorded for TSS measurement. After this, the sample was placed into a furnace at 550 °C for 1 hour, and then allowed to cool in a desiccator. The reduction in sample mass of this process was then recorded for VSS. All samples were taken in triplicate, and the COV for 5 identical samples was within $\pm 5\%$.

3.2.5 Biogas Composition

The composition of biogas was determined using a Shimadzu GC-2010plus with a thermal conductivity detector (TCD). Gas was separated using a select permanent gases/CO₂ (CP7429) column from Agilent and helium was used as carrier gas. The COV was below $\pm 3\%$ for 5 identical samples.

3.5.6 Characterization of the activated carbons

The pore volume, pore radius, and the Brunauer, Emmett and Teller (BET) surface area of the activated carbons were measured using an automatic apparatus (Micromeritics ASAP 2020) at 77 K by nitrogen adsorption and desorption. The measured values are shown in Table 3.3

3.5.7 Size Exclusion Chromatography (SEC)

Size exclusion chromatography (SEC) was carried out using an HPLC (Shimadzu HPLC SPD-20AD) system equipped with 2 columns connected in series (PolySep GFC-P1000 and 4000 Phenomenex), with UV-DAD and refractive index detectors (RID) (Shimadzu). The standards used for molecular weight calibration were the EasiVial polymer standards (Agilent U.S.A.) with molecular weights ranging from 106 g/mol to 1,522K g/mol. The mobile phase was Milli-Q water at a flowrate 1 mL/min, and the analysis time was 30 mins. The COV for 5 identical samples was $\pm 5\%$.

3.5.8 SMP

The identification of small organic compounds known as low molecular weight soluble microbial products (low-MW SMP) was carried out using the following two steps: pre-treatment by an extraction method modified from a solid phase extraction (SPE) procedure (Kunacheva et al., 2017a) followed by analysis using a gas chromatography-

mass spectrometry (GC-MS). In the extraction step the filtered samples (0.45 µm glass fibre filter) were loaded onto 1 or 2 pre-conditioned cartridges connected in series (Waters Oasis®HLB). The cartridges were conditioned using methanol (10 mL), and then washed with ultrapure water (20 mL). The volume of the samples analysed, and the number of cartridges used varied with different types of liquid samples based on their COD concentration; 1 L of the effluent samples and 100 mL of filtered supernatant were used due to the supernatant normally having a higher COD than the effluent samples. The compounds were eluted from the SPE columns with different solvents (methanol, acetone, dichloromethane and hexane) in sequence, and then freeze dried to reduce their volumes, and then analysed using a GC-MS (GCMSQP2010ULTRA Shimadzu). The GC-MS was equipped with an RTX®-5MS (30 m × 0.25 mm ID Restek) column with splitless injection, and was operated at a controlled temperature at 280 °C with Helium as a carrier gas at 1 mL/min. The program running was 7 mins at 50 °C, then increase to 325 °C at a rate of 7°C/min, and then hold for 14 mins. The electron ionisation mode was set at a temperature of 230°C. The selection mass spectra were obtained after 10 mins solvent cut time and with an m/z from 30 to 580, and the identification of chromatographic peaks was based on the NIST11 library (NIST11 library). The level of clarifying “identified” compounds was set at a matching percentage of 80% and higher; whereas, the rest were considered as unknown peaks. In addition, the similarity index, mass spectrum and retention index were also used for the identification of specific compounds.

The SMPs identified were divided into different groups including; alkane, alkene, alcohol, acid, ester, ketone, aldehyde, and aromatic based on their functional groups. The aromatic compounds are compounds that contained phenyl groups, were further divided into N-compounds, sulfur-containing compounds (S-compounds), phenolics, or a combination of all of them. The compounds with a matching percentage of <80% was considered to be an unknown or in the ‘others’ group.

3.5.9 Microbial community

DNA was extracted from the centrifuged pellets of the samples using the MoBio’s PowerSoil® DNA extraction kit (MOBIO, Carlsbad, CA, USA) following the manufacturer’s instructions. The quality of the extracted DNA was checked by determining its absorbance in a NanoDrop One (Thermo Fisher Scientific), and the extracts were stored at -20 °C before sequencing. Polymerase chain reaction (PCR)

amplification was performed by Macrogen Inc. (Seoul, Republic of Korea) using universal prokaryotic primers targeting the V3-V4 region (341F-805R), as described in a previous paper (Takahashi et al., 2014). The MiSeq sequencer at Macrogen Inc. generated paired-end (2×300 bp) 16 rRNA gene sequence data. The raw 16S rRNA gene sequence data was analyzed using the MiSeq SOP pipeline (Kozich et al., 2013) with all default settings. Chimeric sequences, and the sequences assigned to chloroplast, mitochondria, unknown, archaea, and eukaryote were removed for further analysis. The remaining sequences were clustered into operational taxonomic units (OTUs) with 97% nucleotide cutoff.

The 16S rRNA gene sequence datasets used in this study were deposited in GenBank under the accession numbers: MaiInitial1f (SRS2340239), MaiInitial2f (SRS2340240), MaiInitial3f (SRS2340237), Day12CIP051f (SRS2340196), Day12CIP052f (SRS2340248), Day12CIP053f (SRS2340247), Day12CIP501f (SRS2340246), Day12CIP502f (SRS2340245), Day12CIP503f (SRS2340251), Day12Control1f (SRS2340252), Day12Control2f (SRS2340250) and Day12Control3f (SRS2340249).

3.5.10 CIP

The CIP concentration was measured using a triple quadrupole mass spectrometer equipped with an Agilent Jet Stream Electrospray ionization injector (AJS-ESI) (LCQQQ 6460 Agilent). The instrument was setup as follows; gas temperature 300 °C, gas flow 10 L/min, nebulizer 25 psi, sheath gas temperature 250 °C, sheath gas flow 10 L/min. A Kinetex 2.6 µm Bi-phenyl column (100×2.1 mm - Phenomenex USA) was used for chromatographic separation with a mobile phase of 0.1% HCOOH in water (A), and 0.1% HCOOH in acetonitrile (B) at a gradient of 10–90 % B within 6 min. The COV for five identical samples was within ±5%.

3.5.11 CIP intermediates

The identification of CIP intermediates was done in an LC-MS/MS similar to the analysis of CIP in 3.5.10, except that the instrument was used in scanning mode instead of M2M mode, and a change in the mobile phase program. The mobile phase for scanning was set at a gradient of 10-90% of HCOOH (0.1%) in acetonitrile, with a total analysis time of 12 mins at a flow rate of 0.2 ml/min. The scan of CIP intermediates was conducted in a GC-MS with a pre-treatment of the sample using SPE extraction from 1 L of effluent. The SPE cartridge was eluted with 2 ml of individual solvents (methanol,

acetone, dichloromethane and hexane), and the eluents were analysed separately by the GC-MS using the method for low-MW SMPs described in 3.5.8.

3.5.12 Adsorption onto biomass

The CIP adsorbed onto the sludge was extracted using the procedure described as follows; the mixed liquor sludge sample from the AnMBR was centrifuged at 4000 rpm for 5 min. After this the pellet was separated from the liquid phase and washed with Milli-Q water once. The pellet was then freeze-dried (under vacuum at -80 °C) for 24h before being weighed and then ground to a fine powder using a mortar and pestle, and 5ml of a water:methanol (90:10 by volume) mixture was added at pH 11 to extract the CIP. The solvent mixture (methanol and water), and the pH conditions for extraction were chosen after previous trials of varying the pH and water to methanol ratio (results not showed) following (Okuda et al., 2009). The slurry containing the powdered freeze-dried sample and the water:methanol (90:10) mixture were vortexed and then ultrasonicated for 15 mins at 30°C. After this, the sample was centrifuged, and the supernatants were transferred to a glass tube, while the remaining solids underwent another extraction step. Finally, the two extraction samples were combined and filtered through a 0.2 µm filter before analysis by LC-MS/MS as described in Section 3.5.10.

3.6 Mass balance on the removal CIP from AnMBR

The mass balance to determine the fate of CIP removed from the AnMBR was calculated based on the following equation:

$$\text{Influent (mg/d)} = \text{sorption (mg/d)} + \text{effluent (mg/d)} + \text{degradation/transformation (mg/d)} + \text{waste sludge (mg/d)}$$

$$\text{Degradation/transformation} = \text{influent} - \text{sorption} - \text{effluent} - \text{waste sludge}$$

$$\text{Sorption onto biomass} = q \times \text{TSS} \times V_{\text{reactor}}$$

$$\text{Waste sludge} = q \times \text{TSS} \times V_{\text{sludge withdraw}}$$

Where q is the amount of CIP sorbed onto the sludge (mg/g). The equation assumed that the CIP added to the AnMBR was either degraded/transformed, sorbed to sludge, exited in the final effluent, or was discharged with the gradually withdrawn sludge. No abiotic reduction of CIP was shown in the equation since preliminary experiments revealed no degradation of CIP occurred by volatilization, or adsorption to the wall of the reactor.

3.7 Theoretical COD calculation for low MW-SMPs compounds

The theoretical COD for VFAs, which was modified from a past study (Aquino and Stuckey, 2004b), and low MW-SMPs compounds were calculated as below. The conversion ratio of converting SMPs to COD was calculated and shown in Table 3.4

$$\text{COD}_{\text{VFA}} = 0.35 \times (\text{formate}) + 1.07 \times (\text{acetate}) + 1.51 \times (\text{propionate}) + 1.82 \times (\text{butyrate} + \text{isobutyrate}) + 2.04 \times (\text{valerate} + \text{isovalerate})$$

$$\text{COD}_{\text{low MW_SMP}} = \text{COD}_{\text{alkanes}} + \text{COD}_{\text{alkenes}} + \text{COD}_{\text{esters}} + \text{COD}_{\text{phenols}} + \text{COD}_{\text{N-compounds}} + \text{etc.}$$

$$\text{COD}_{\text{alkane}} = 3.44\text{-}3.49 \text{ C}_{\text{alkanes}} \text{ (used for alkane from C10- C40)}$$

$$\text{COD}_{\text{alkene/cycloalkanes}} = 3.43 \text{ C}_{\text{alkenes/cycloalkane}}$$

$$\text{COD}_{\text{phenols}} = 2.52 \text{ C}_{\text{p-cresol}} + 2.95 \text{ C}_{\text{Dibutylphenol}}$$

$$\text{COD}_{\text{N-compounds}} = 1.86\text{-}3.01 \text{ C}_{\text{N-compounds}}$$

Table 3.4 Illustration of reactions and conversion ratios from SMP concentration to COD concentration.

Compounds	Reaction	Conversion ratio [*]
Phenols	$2 \text{ C}_7\text{H}_8\text{O} + 17 \text{ O}_2 = 14 \text{ CO}_2 + 8 \text{ H}_2\text{O}$	2.52
	$\text{C}_{14}\text{H}_{22}\text{O} + 19 \text{ O}_2 = 14 \text{ CO}_2 + 11 \text{ H}_2\text{O}$	2.95
Alkane	$2 \text{ C}_{10}\text{H}_{22} + 31 \text{ O}_2 = 20 \text{ CO}_2 + 22 \text{ H}_2\text{O}$	3.49
	$2 \text{ C}_{20}\text{H}_{42} + 61 \text{ O}_2 = 40 \text{ CO}_2 + 42 \text{ H}_2\text{O}$	3.46
	$2 \text{ C}_{40}\text{H}_{82} + 121 \text{ O}_2 = 80 \text{ CO}_2 + 82 \text{ H}_2\text{O}$	3.44
Alkenes/cycloalkane	$\text{C}_n\text{H}_{2n} + 3n/2 \text{ O}_2 = n \text{ CO}_2 + n \text{ H}_2\text{O}$	3.43
Ester	$\text{C}_{19}\text{H}_{36}\text{O}_2 + 27 \text{ O}_2 = 19 \text{ CO}_2 + 18 \text{ H}_2\text{O}$	2.92
	$2 \text{ C}_{19}\text{H}_{38}\text{O}_2 + 55 \text{ O}_2 = 38 \text{ CO}_2 + 38 \text{ H}_2\text{O}$	2.95
	$2 \text{ C}_{20}\text{H}_{30}\text{O}_4 + 51 \text{ O}_2 = 40 \text{ CO}_2 + 30 \text{ H}_2\text{O}$	2.44
N-compounds	$\text{C}_{11}\text{H}_{18}\text{N}_2\text{O}_2 + 13 \text{ O}_2 = 11 \text{ CO}_2 + 6 \text{ H}_2\text{O} + 2 \text{ NH}_3$	2.59
	$\text{NH}_3 + 2 \text{ O}_2 = \text{HNO}_3 + \text{H}_2\text{O}$	

	$2 \text{ C}_{10}\text{H}_{14}\text{N}_2\text{O}_3 + 21 \text{ O}_2 = 20 \text{ CO}_2 + 8 \text{ H}_2\text{O} + 4 \text{ NH}_3$ $\text{NH}_3 + 2 \text{ O}_2 = \text{HNO}_3 + \text{H}_2\text{O}$	2.23
	$\text{C}_7\text{H}_{10}\text{N}_2\text{O}_2 + 7 \text{ O}_2 = 7 \text{ CO}_2 + 2 \text{ H}_2\text{O} + 2 \text{ NH}_3$ $\text{NH}_3 + 2 \text{ O}_2 = \text{HNO}_3 + \text{H}_2\text{O}$	2.29
	$\text{C}_{14}\text{H}_{22}\text{N}_2\text{O}_2 + 17 \text{ O}_2 = 14 \text{ CO}_2 + 8 \text{ H}_2\text{O} + 2 \text{ NH}_3$ $\text{NH}_3 + 2 \text{ O}_2 = \text{HNO}_3 + \text{H}_2\text{O}$	2.69
	$\text{C}_{12}\text{H}_{12}\text{N}_2\text{O}_5 + 11 \text{ O}_2 = 12 \text{ CO}_2 + 3 \text{ H}_2\text{O} + 2 \text{ NH}_3$ $\text{NH}_3 + 2 \text{ O}_2 = \text{HNO}_3 + \text{H}_2\text{O}$	1.86
	$2 \text{ C}_{11}\text{H}_{12}\text{N}_2\text{O}_2 + 23 \text{ O}_2 = 22 \text{ CO}_2 + 6 \text{ H}_2\text{O} + 4 \text{ NH}_3$ $\text{NH}_3 + 2 \text{ O}_2 = \text{HNO}_3 + \text{H}_2\text{O}$	2.43
	$2 \text{ C}_{14}\text{H}_{16}\text{N}_2\text{O}_2 + 31 \text{ O}_2 = 28 \text{ CO}_2 + 10 \text{ H}_2\text{O} + 4 \text{ NH}_3$ $\text{NH}_3 + 2 \text{ O}_2 = \text{HNO}_3 + \text{H}_2\text{O}$	2.56
	$2 \text{ C}_8\text{H}_7\text{NO} + 17 \text{ O}_2 = 16 \text{ CO}_2 + 4 \text{ H}_2\text{O} + 2 \text{ NH}_3$ $\text{NH}_3 + 2 \text{ O}_2 = \text{HNO}_3 + \text{H}_2\text{O}$	2.53
	$\text{C}_8\text{H}_7\text{N} + 9 \text{ O}_2 = 8 \text{ CO}_2 + 2 \text{ H}_2\text{O} + \text{NH}_3$ $\text{NH}_3 + 2 \text{ O}_2 = \text{HNO}_3 + \text{H}_2\text{O}$	3.01

Note: Conversion ratio is the ratio to convert the concentration of SMP compounds to COD concentration.

3.8 Biological methane potential

The cumulative CH_4 production in the batch experiment for anaerobic digestion was fitted to a modified Gompertz equation (Eq. 1) using Sigma Plot software. From this, three parameters were calculated, including the ultimate CH_4 yield (mL) (P), the maximum CH_4 production rate (mL/d) (R_m), and the lag phase (d) (λ); these were needed for the prediction of methane production. The other parameter in the equation was M(t), the cumulative CH_4 production (mL) at time t (d).

$$M(t) = P \times \exp \left\{ -\exp \left[\frac{R_m \times e}{P} \times (\lambda - t) + 1 \right] \right\} \quad (\text{Eq. 1})$$

$$R_{m(\text{relative})} = R_m / R_{m \text{ ave } (\text{control})} \times 100 \quad (\text{Eq. 2})$$

The relative maximum CH_4 production rate $R_{m(\text{relative})}$ (mL/d) for each R_m value was based on the average R_m of the control samples, which was set equal to 100, by Equation

2. The 20, 50 and 80% inhibition concentrations (IC_{20} , IC_{50} , IC_{80}) were determined by plotting R_m as a function of initial CIP concentration. Curve fitting was done using the website mycurvefit.com, and the inhibition concentrations were calculated based on the fitting equation.

3.9 Statistical analysis

Statistical testing of differences between various system parameters (e.g., biogas production, COD, VFA) was performed using the Student's t-test with a threshold at $p = 0.05$.

CHAPTER 4

Effects of different concentrations of CIP on anaerobic digestion²

Outline:

4.1 Introduction

4.2 Material and methods

4.3 Results and discussion

4.4 Conclusions

4.1 Introduction

The worldwide presence of pharmaceuticals in aqueous environments has been documented to increase the risk of spreading antibiotic resistance genes, and raises the potential risk to human health and the ecosystem (Heberer, 2002). The quinolone antibiotics are one of the most common antibiotic groups, and are used to treat a variety of bacterial infections in humans for decades (Emmerson and Jones, 2003). Due to the ingestion and excretion process of humans and other organisms, a large amount of these compounds is discharged into the sewerage system in all countries, and then transport to WWTPs. In sewage, CIP concentrations are generally low ($<1.9 \mu\text{g/L}$) (He et al., 2015), although hospital wastewaters can often have much higher concentrations of up to $101 \mu\text{g/L}$ (Lindberg et al., 2004). However, the effluent from an Indian Plant, Patancheru Enviro Tech Ltd, primarily treating wastewater from 90 bulk drug manufacturers, contained CIP concentrations up to $28\text{--}31 \text{ mg/L}$ (Larsson et al., 2007). The incomplete treatment of CIP in WWTPs results in surface, ground and drinking waters nearby to be contaminated with CIP, and concentrations as high as 6.5 mg/L and 2.5 mg/L were found in lake and river waters, respectively (Fick et al., 2009). This

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concentration exceeds human therapeutic blood plasma concentrations (2.5 mg/L) (Schulz and Schmoldt, 2003). In addition, the incomplete treatment of CIP as well as other antibiotics in WWTP was reported in many studies from various countries including Australia, France, Spain, and Korea (Dinh et al., 2017, Rodriguez-Mozaz et al., 2015, Sim et al., 2011, Watkinson et al., 2009), with concentrations of CIP in effluents ranging from ng/L to µg/L (up to 3403 ng/L). As a consequence, a large amount of antibiotics is deposited into the aquatic environment, and typically downstream of the Fontenay-les-Briis WWTP in France, up to 1523 ng/L was detected, or in Australia surface waters a maximum of 1300 ng/L was detected (Watkinson et al., 2009).

Based on the potential environmental risk assessment established by the calculation of risk quotients of the thirteen antibiotics, CIP is one of the antibiotics that poses a high ecological risk to most sensitive aquatic organisms including *Vibrio fischeri*, *Microcystis aeruginosa* and *Synechococcus leopoliensis* (Zhang et al., 2012). The effects of CIP on organisms occurs at quite low concentrations; the EC₅₀ toxicity values were found to be 17 and 203 µg/L for *Microcystis aeruginosa* and *Lemna minor*, respectively (Robinson et al., 2005), and genotoxicity, and the transfer of resistance between different bacterial species were induced at 5–10 µg/L (Beaber et al., 2004, Hartmann et al., 1998, Hartmann et al., 1999). In addition, for in-vitro activity 31% of the 362 strains of anaerobic bacteria were susceptible to CIP, with tentative breakpoints (a concentration used to specify susceptible, intermediate or resistant in the susceptibility testing) of 1 µg/mL (Sutter et al., 1985). Hence, CIP can be toxic and pose ecological risks at concentrations commonly found in the environment. Hence, it would be desirable to remove high levels of CIP in WWTPs to prevent the ecological risks of spreading CIP to the aquatic environment.

A few studies have investigated the treatment of hospital wastewater containing CIP using an aerobic membrane bioreactor (MBR) (Nguyen et al., 2017), and a two stage and three stage MBR (Hamjinda et al., 2017). However, with recently increasing interest in anaerobic systems to treat wastewater, including the UASB, and the AnMBR (Chong et al., 2012b, Smith et al., 2012), and with the ubiquitous presence of CIP in the environment, an investigation into the effect of CIP on anaerobic digestion would seem timely. Moreover, with the recent development of analytical techniques for measuring and identifying low molecular weight (MW) soluble microbial products (SMPs) (<580

Da) (Kunacheva et al., 2017a), this will be the first time we can truly understand what compounds (other than VFAs) are released during CIP exposure. This information, together with a general study on the effect of CIP on COD removal, methane production and VFA accumulation as well as the impact of CIP exposure on microbial ecology will be investigated in this study to provide greater insights on the effect of CIP on anaerobic digestion. Finally, the removal of CIP in anaerobic digestion will also be studied to determine the potential of CIP removal during anaerobic condition.

4.2 Experimental and analytical details

The experiment was done in serum bottles as described in part 3.3.1. The measurement of COD, VFA, CIP, low MW SMPs compounds in the supernatant, the amount of CIP absorbed onto the sludge, the scan of CIP intermediate compounds and the microbial community were described in Chapter 3.5.

The ultimate CH_4 yield (mL) (P), the relative maximum CH_4 production rate (mL/d) ($R_{m \text{ (relative)}}$), the maximum CH_4 production rate (mL/d) (R_m), the lag phase (d) (λ) and the 20, 50 and 80% inhibition concentrations (IC_{20} , IC_{50} , IC_{80}) were calculated as described in Part 3.8.

4.3 Results and discussion

4.3.1 Effect of increasing CIP concentrations on anaerobic digestion

4.3.1.1 Effect on biogas production

Cumulative methane production (Fig. 4.1.a) indicated that the rate and levels of methane production decreased as the CIP concentration increased, although methanogenesis still occurred, even at 50 mg/L. From the control to CIP50 final CH_4 production decreased from 115.3 mL to 66.7 mL, suggesting that the methanogenic community was sensitive to CIP, and a significant effect was observed even at 0.5 mg/L ($p < 0.05$), although 0.05 mg/L was not significant.

Mathematically, the degradation rate of compounds can be described by a differential equation. Assuming biogas production is proportional to microbial activity, the modified Gompertz equation (Eq. 1) was used to predict methane production, and was

originally developed to describe the growth of bacteria in batch mode (Lay et al., 1997). The Gompertz model was fitted to methane production to evaluate the parameters influencing anaerobic digestion, and the fit was good ($R^2 \geq 0.97$). The kinetic constants calculated including ultimate production (P), maximum production rate (R_m) and lag time (λ) are presented in Table 4.1. The values of P and R_m show significant differences between the CIP reactors and the control ($p < 0.05$) from concentrations of 0.5 mg/L and higher. The Table also shows the lag phase with 4 negative values, which due to the model fitting can be judged as an inaccurate estimation of 0.

Table 4.1 Calculated fitting of experimental values to the three-parameter Gompertz equation

Sample	P (mL)	R_m (mL/d)	λ (d)	R^2
Control	116.05 $\pm$ 3.49	11.17 $\pm$ 0.22	< 0	0.99
CIP0.05	111.54 $\pm$ 0.29	11.00 $\pm$ 0.07	0.14 $\pm$ 0.04	1.00
CIP0.5	107.41 $\pm$ 2.04*	10.07 $\pm$ 0.30*	0.12 $\pm$ 0.07	1.00
CIP2.5	102.72 $\pm$ 2.62*	7.90 $\pm$ 0.03*	< 0	0.99
CIP5	98.35 $\pm$ 0.21*	6.69 $\pm$ 0.26*	< 0	0.98
CIP50	78.89 $\pm$ 15.21*	4.37 $\pm$ 0.18*	< 0	0.97

Note: the parentheses () show the p value for the t-test compared with values obtained in the Control bottle. P: ultimate CH₄ yield (mL), R_m : the maximum CH₄ production rate (mL/d), λ : the lag phase (d); R^2 : square of coefficient of correlation; *: statistical significant.

From this analysis, the relative R_m s were used to predict the inhibition concentrations IC₂₀, IC₅₀, and IC₈₀ (Fig. 4.1.b); these values are useful to compare the resistance of this microbial community to other studies, as well as to predict its behaviour at different concentrations. The calculated values for CIP were IC₂₀ = 0.11; IC₅₀ = 4.8; IC₈₀ > 42.94 mg CIP/L, and showed that the culture in our study was more sensitive to CIP compared to another study which only showed significant inhibition at 80-100 mg CIP/L (Liu et al., 2013c). The IC₅₀ value of CIP is considered to be in the same range as the most toxic chlorophenol, pentachlorophenol, which causes 50% biogas reduction in the 0.5–10 mg/L range (Chen et al., 2008), although the mechanism by which N-substituted aromatics and chlorinated compounds cause bacterial toxicity is different. In general,

N-substituted aromatics, such as nitrobenzenes, aminophenols, etc., are reactive toxicants (Blum and Speece, 1991) which interact with enzymes, or interfere with metabolic pathways. In contrast, the toxicity of chlorophenols is induced by disturbing the proton gradient through membranes, and hence the energy transduction in cells (Chen et al., 2008). CIP contains three nitrogen atoms in its structure, with two tetra amines and one secondary amine. Like other antibiotics in the quinolone class, CIP can form a stable complex between the CIP-topoisomerase enzyme, and cleaved DNA. This enzyme is involved in replicating and repairing DNA, which means bacteria cannot repair themselves, or reproduce. This will prevent DNA replication, inhibit DNA synthesis, and consequently leads to bacteriostasis and cell death.

Figure 4.1 Effect of varying concentrations of CIP over time (day) on (a) total methane production and (b) relative maximum CH₄ production rate (mL/d).

Note: $R_m \text{ (relative)} = R_m / R_{m \text{ ave (control)}} * 100$, where $R_m \text{ (relative)}$ is the relative maximum CH_4 production rate (mL/d), R_m is the maximum CH_4 production rate (mL/d), $R_{m \text{ ave (control)}}$ is the average maximum CH_4 production rate (mL/d) in the control reactor. Control, CIP0.05, CIP 0.5, CIP2.5, CIP5, and CIP50 represents the reactor with CIP concentration of 0, 0.05; 0.5; 2.5; 5; 50 mg/L, respectively. Each data point represents the average of three replicates, while the error bars represent the standard deviation of the measurements.

4.3.1.2 Effect on COD removal, VFA and SMP accumulation

The effect of CIP on the accumulation of VFAs (Fig. 4.2), COD removal (Fig. 4.3), and low-MW SMPs (Table 4.2) is not unlike its effect on biogas production; a significant effect on these parameters occurred at concentrations of 0.5 mg/L and higher. The decrease in methane production may have resulted from the direct inhibition of methanogens, or from disrupting fermentation which interrupts VFA production. However, in this study an increase in VFAs was observed (Fig. 4.2), which means the second hypothesis does not hold. On day 12, acetate dominated the VFAs in all samples, and the total VFAs were higher at higher CIP concentrations. After this time the VFAs were consumed, and their concentration decreased on days 24 and 36, although with CIP50 it increased again after 36 days. The change in COD removal was observed with a large reduction on day 12 d, but not for days 24 and 36 (except for CIP50). The COD removal is explained by the mass balance of C-compounds where $\text{COD}_{\text{initial}} = \text{COD}_{\text{methane}} + \text{COD}_{\text{supernatant}}$. The COD removal was linked with the COD concentration that goes to the gas phase ($\text{COD}_{\text{methane}}$). After 12 days (except for CIP50), the methane production reduced significantly, and therefore COD removal reduced.

Figure 4.2 Effect of increasing concentrations of CIP over time (day) on VFA accumulation. Control, CIP0.05, CIP 0.5, CIP2.5, CIP5, and CIP50 represented the reactor with CIP concentrations of 0, 0.05; 0.5; 2.5; 5; 50 mg/L, respectively. Acetic acid, propionic acid, Isobutyric acid, Butyric acid, Isovaleric acid and Valeric acid are VFAs.

With the release of low-MW SMPs, the chromatogram of the SMPs in the control, CIP0.05 and CIP0.5 were similar except for the formation of 5-methyl- 1H-Benzotriazole in CIP0.5. The number of peaks identified for all samples is summarised in Table 4.2, and are divided into generic groups of organics, including alkanes, alkenes, alcohols, acids, ester, ketones, aldehydes, and aromatics, which could be N-compounds, S-compounds, phenolics, or a combination of all of them. The number of aromatic compounds detected increased at high CIP concentrations, and it is noteworthy that this increase resulted in an increase in N-compounds, whereas S- and phenol compounds did not change significantly. Seven N-compounds were found in the Control reactor, and five were identified with similarities greater than 90%, including Benzenamine, N-ethyl; Benzothiazole; Benzothiazole, 2-(methylthio)-; 2(3H)-Benzothiazolone, and; 1-(Benzthiazol-2-ylthio)-propan-2-one. At CIP2.5 and higher, more N-compounds appeared such as Indole; 1-isocyanato-2-methylbenzene; 1-(Benzthiazol-2-ylthio)-

propan-2-one; 2,5-Piperazinedione, 3-(phenylmethyl). This increase is probably the result of stress caused by CIP as it results in bacteriostasis and cell death, as describe previously, or it could be the products of CIP metabolism. Indolic compounds such as Indole are products of aromatic amino acid metabolism (from tryptophan) (Smith and Macfarlane, 1996). Some N-compounds were also found in a study on the effect of different HRTs on AnMBRs (Kunacheva et al., 2017c), such as Indole and 5-methyl-1H-Benzotriazole. However, the microorganisms which produce these compounds are unknown, as well as why they are produced in the culture.

Figure 4.3 Effect of CIP on COD removal. Control, CIP0.05, CIP 0.5, CIP2.5, CIP5, and CIP50 represented the reactor with CIP concentrations of 0, 0.05; 0.5; 2.5; 5; 50 mg/L, respectively. Each bar represents the average of three replicates, while the Error bars represent the standard deviation of the measurements.

Another interesting observation was the dominance of p-Cresol at CIP concentrations of 2.5 mg/L (Fig. 4.4); p-cresol was found to be from 5 to 8 times higher than the second most abundant SMP (5-methyl-1H-Benzotriazole). In addition, some aromatic compounds were found in all samples in which 2,4-bis(1,1-dimethylethyl)-phenol and 2,6-bis(1,1-dimethylethyl)-phenol were widely found. Similarly to indole, p-cresol and other phenolic compounds are produced from aromatic amino acid metabolism, but unlike indole and indolic compounds which are produced from tryptophan, phenolic compounds are produced from tyrosine (Smith and Macfarlane, 1996).

Table 4.2 Number of organic compounds found in the supernatant of the serum bottles

Samples	Number of low-MW SMP compounds								
	Total	Alkane, alkene	Aromatic compounds	N - compounds	S - compounds	Ester	Aldehyde, ketone, alcohol	Acid	Other
Control	41	18	10	7	4	7	4	0	2
CIP0.05	40	19	9	6	4	8	3	0	1
CIP0.5	37	18	9	7	4	7	3	0	0
CIP2.5	43	6	21	17	6	10	2	0	4
CIP5	41	17	16	13	5	4	2	0	2
CIP50	79	20	32	31	3	1	3	18	5

Note: All N-compounds and S-compounds are aromatic compounds. Control, CIP0.05, CIP 0.5, CIP2.5, CIP5, and CIP50 represented reactor with CIP concentration of 0, 0.05; 0.5; 2.5; 5; 50 mg/L, respectively.

Figure 4.4 Chromatograms of organic compounds found at various CIP concentrations. Control, 0.05, 0.5, 2.5, 5, and 50 represented the reactor with CIP concentrations of 0, 0.05; 0.5; 2.5; 5; 50 mg/L, respectively.

Furthermore, the appearance of various fatty acids (excluding VFAs) at CIP50 only was remarkable, and included phenolic acids such as benzeneacetic acid, hydrocinnamic acid and long chain fatty acid (octanoic acid, nonanoic acid, decanoic acid, etc.). Conversely, esters, which were found at high percentages in the Control, and CIP0.05 to CIP2.5, decreased at high CIP concentrations. Some N-compounds from the inlet feed

could not degrade at high CIP concentrations, and were still present in the supernatant after 45 days, including hexahydro-pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-pyrrolo[1,2-a]pyrazine-1,4-dione, and, hexahydro-3-(2-methylpropyl)-pyrrolo[1,2-a]pyrazine-1,4-dione.

The identity of all the SMP solutes found in our study are shown in Table S4.2, and total 68 compounds. The high concentration of SMPs in the effluent could be toxic to the eco-system and human health as they contained a large number of aromatic and N-compounds; a few SMPs compounds such as Indole, p-cresol and other phenolic compounds, etc. are known to be carcinogenic (Nowak and Libudzisz, 2006). Moreover, SMPs can act as precursors for disinfection by-products after wastewater disinfection that could convert SMPs into more toxic substances (Zhang et al., 2015). Some of these by-products are considered genotoxic, mutagenic and/or carcinogenic with obvious adverse effects on human health (Richardson et al., 2007). Nitrogen-containing disinfection by-products are more toxic than carbon-containing disinfection by-products, for example, the haloacetamides are approximately 140 times more cytotoxic, and 12 times more genotoxic than any of the 5 regulated haloacetic acids (Plewa et al., 2007); dibromonitromethane is approximately 83 times more cytotoxic and 67 times more genotoxic than dibromoacetic acid (Plewa et al., 2002, Plewa et al., 2004).

4.3.1.3 The impact of CIP on microbial community structure

16S rRNA gene-based community analysis was performed on the inoculum and communities (Control, CIP0.5 and CIP50) sampled from serum bottles on day 12 when methanogenesis was active. The microbial community structure among the communities was evaluated by non-metric multidimensional scaling (nMDS) analysis (Fig. 4.5) using microbial community composition at OTU level. The inoculum, Control, and CIP0.5 communities clustered closely together, whereas the CIP50 communities were differentiated significantly from the other communities. The pairwise similarity using the Bray-Curtis distance was 0.83 ± 0.02 (Control vs. CIP0.5) and 0.59 ± 0.02 (Control vs. CIP50), suggesting that 50 mg/L of CIP significantly altered the community phylogenetic structure.

Figure 4.5 nMDS of all Operational Taxonomic Units (OTU) generated for digesters for Initial, Control, reactor with 0.5 mg/L (CIP0.5) and reactor with 50 mg/L (CIP50) on day 12. Ellipses represent 95% confidence intervals for the three points.

Fig. 4.6.a shows major bacterial and archaeal phyla with > 1% of relative abundance on average. Five major phyla were predominant in the inoculum communities (sludge taken from AnMBRs), and were *Firmicutes* (27.4%), *Bacteroidetes* (17.8%), *Synergistetes* (17.7%), *Candidatus_Saccharibacteria* (13.5), and *Chloroflexi* (11.8). The principal phyla detected were found by other authors, and Firmicutes was also the dominant group in a UASB (Li et al., 2015) and AnMBR (Ng et al., 2015). After 12 days, there was a shift in the distribution of these phyla, however, in the control reactor the order of these phyla stayed the same. Consistent with the beta-diversity results shown in Fig. 4.5, 50 mg/L of CIP could cause dramatic shifts in microbial community composition at the phylum level. While CIP reduced the relative abundance of Firmicutes (14.5% in CIP50) and Bacteroidetes (12.5%), Synergistetes and Candidatus Saccharibacteria increased significantly to 22.6% and 25.7%, respectively.

Figure 4.6 The effect of CIP addition on bacteria and archaea at class level for Initial, Control, reactor with 0.5 mg/L (CIP0.5) and reactor with 50 mg/L (CIP50) on day 12 on (a) phylum and (b) genus level. The major phyla and genera with an average of > 1% of relative abundance are shown. The number 1, 2, 3 showed the replicate of the samples.

At genus level, *Trichococcus* (18.2%) dominated the Control communities, followed by *Methanothrix* (10.6%), *Syntrophobacter* (2.7%), *Melioribacter* (2.2%), and *Geobacter* (1.3%). The change in genus level with low (0.5 mg/L) or high (50 mg/L) concentrations of CIP could be divided into 3 general groups. The first group represents inhibition at

low CIP concentrations (from CIP0.5) and include *Syntrophobacter*, *Aminomonas*, *Melioribacter*, *Geobacter*, and *Methanothrix*; *Syntrophobacter* are involved in acetogenesis, which anaerobically degrade propionate to acetate in the presence of methanogens. The under-representation of *Syntrophobacter* was in a good agreement with the accumulation of propionate at 50 mg/L of CIP (Fig. 4.2). *Methanothrix* are a genus of *Methanosarcinales* that are typical acetoclastic methanogenic archaea. Since members of the *Methanosarcinales* are capable of acetoclastic methanogenesis (Vanwonterghem et al., 2016), the inhibition on the growth of *Methanothrix* presumably resulted in the significant reduction of methane production at 0.5–50 mg/L of CIP.

Trichococcus, *Anaeroarcus*, *Macellibacteroides* and *Sedimentibacter* were reduced significantly in the communities exposed to 50 mg/L of CIP, although those genera were comparable between the Control and CIP0.5 communities. *Trichococcus* can ferment glucose, producing lactate, formate, acetate and ethanol (Liu et al., 2002). *Anaerococcus* isolated from clinical settings were resistant to toxic compounds such as SPS, tetracycline, erythromycin and clindamycin, clindamycin, levofloxacin and ceftazidime (Murphy and Frick, 2013). Notably, *Anaerococcus* are capable of utilizing peptone and amino acids as their major energy source and producing butyrate as metabolites (Ezaki et al., 2001). Hence, our results and the previously described physiology of *Trichococcus* and *Anaeroarcus* collectively implied that the inhibition of these genera under the CIP exposure might also contribute to the dramatic decrease in methane production

Methanobacterium, *Treponema*, *Clostridium*, *Desulfovibrio*, *Bacteroides*, *Methanolinea*, *Romboutsia* and *Petrimonas* were significantly enriched with 0.5–50 mg/L of CIP. This group of organisms include hydrolytic bacteria, fermentative, and sulfate-reducing bacteria with representatives such as *Bacteroides*, *Petrimonas*, and *Desulfovibrio*, respectively. Methane can be produced from acetate via the two pathways: acetoclastic cleavage and non-acetoclastic oxidation (Petersen and Ahring, 1991, Schnürer et al., 1994). The first methane production pathway is associated with acetoclastic methanogenic archaea, *Methanothrix*, the inhibition of which under the CIP exposure was observed with the sequencing and experimental data (Figs. 4.2 and 4.6). *Methanobacterium* are a genus of *Methanobacteriales* that can reduce CO using

molecular hydrogen (hydrogenotrophic) and produce methane (Hattori et al., 2000, Petersen and Ahring, 1991, Schnürer et al., 1997). *Clostridium* are known to be acetate-oxidizing bacteria coupled to hydrogenotrophic methanogenesis. Accordingly, it is likely that the hydrogenotrophic methane production pathway might be less disturbed, even at 50 mg/L of CIP, compared to those with the aceticlastic methanogenesis pathway.

4.3.2 Removal of CIP in the batch test

The average CIP removal ranged from 85% (70-95%) at 12 days, to 91% (86-100%) after 45 days, with increasing CIP concentrations lowering the removal rate of the reactor (Fig. 4.7). In addition, in the final experiment the amount of CIP adsorbed onto the biomass was measured (Table 4.3), and showed that this was strongly dependent on the added concentration of CIP. At the lowest CIP concentration, 2.6 ± 0.3 mg CIP /kg dry weight was detected; this value was consistent with another study on anaerobic digested sludge of 0.5-4.8 mg/kg dry weight (Lindberg et al., 2005). In this study, at high applied concentrations of CIP, adsorption increased dramatically, reaching 462 mg/kg. These values were much higher than the maximum number found in the literature of 16 mg/L in biosolids waste in Canada (Guerra et al., 2014) because it is rare to encounter these concentrations in the environment compared to anaerobic digestion. In addition, a mass balance on the amount of CIP absorbed onto the biomass showing that CIP removal by adsorption accounted for 8.4 to 47.4% removal. The remaining part could be from biodegradation or abiotic removal such as volatile, hydrolysis/photolysis. However, the low Henry's Law constants 5.09×10^{-19} (atm·m³/mol), of CIP indicate that it is not easy to volatilize, and a high MW (331.35) and the presence of two ionizable functional groups (a carboxylic group and a piperazinyl group), suggested that it is not easy to hydrolyse or degrade due to photolysis. Hence, the remaining fraction of the CIP removed could be through biodegradation. However, when the samples were scanned by LC-MS/MS to detect any CIP biotransformation products based on a number of M/z compounds reported in several papers (Hamjinda et al., 2017, Liao et al., 2016), no intermediate products were found. Despite this observation, the increase in some N-compounds detected by GC-MS shown as low-MW SMPs (Table 4.2) could be products from CIP metabolism.

Table 4.3 Amount of CIP adsorbed onto biomass

	CIP0.05	CIP0.5	CIP2.5	CIP5	CIP50
CIP on biomass (mg/kg)	2.6 ± 0.3	27.0 ± 10.0	78.1 ± 3.7	113.1 ± 14.6	461.8 ± 67.7
Contribution to total removal (%)	47.4	49.1	28.4	20.6	8.4

The sorption of CIP onto sludge can either be adsorption or absorption, however, adsorption would be the most plausible sorption mechanism in this case. The bioaccumulation of solutes into fatty tissue is strongly dependent on its hydrophobicity, and compounds are removed by hydrophobic reactions between the compound and the cell membrane of microorganisms, or the lipid fraction of the sewage. The octanol-water partition coefficient (K_{ow}) represents the ability of a solute to absorb, with a $\log K_{ow} < 2.5$ assumed to be poorly absorbed to sewage sludge (Rogers, 1996), and hence CIP with a $\log K_{ow} = 0.28$ should be poorly absorbed into anaerobic sludge. However, despite being quite hydrophilic with a low K_{ow} , CIP is readily adsorbed to sludge via electrostatic interactions between positively charged amino groups, and the negatively charged cell surface of micro-organisms (Ternes et al., 2004).

Figure 4.7 CIP removal in a batch reactor with various concentrations of CIP addition (from 0-50 mgCIP/L). The columns of red, orange and green show the samples taken on days 12, 24 and 36, respectively. Each bar represents the average of three replicates, while the Error bars represent the standard deviation of the measurements.

4.4 Conclusions

The antibiotic CIP at environmentally relevant levels (≥ 0.5 mg/L) caused significant perturbations to anaerobic digestion, with reductions in biogas production, and organic matter removal, and increases in VFA and SMP production. An increase in the number and concentration of aromatic compounds with the predominance of p-cresol from 2.5 mg CIP/L, and large quantities of fatty acids in 50 mg CIP/L were also observed. 16S rRNA gene-based community profiling revealed a significant decrease in *Syntrophobacter* and *Methanothrix* in the CIP-exposed communities, which are known to be associated with acetogenesis and acetoclastic methanogenesis, respectively.

CHAPTER 5

The effect of CIP on an AnMBR

Outline:

5.1 Introduction

5.2 Material and methods

5.3 Results and discussion

5.4 Conclusions

5.1 Introduction

CIP was demonstrated to be genotoxic, and resistance transferable between different bacterial species was induced at CIP concentrations as low as 5–10 µg/L (Beaber et al., 2004, Hartmann et al., 1998, Hartmann et al., 1999). CIP is one of the antibiotics that pose a high ecological risk (risk quotients $RQ > 1$) to the most sensitive aquatic organisms (Zhang et al., 2012), and influence algal community structure at concentrations from 0.012- 5 µg/L (Wilson et al., 2003). They also caused a change in the numbers and species abundance of phytoplankton and zooplankton, and caused fish kills at concentrations of 60-100 µg/L and higher, when combined with ibuprofen and fluoxetine (60-100 µg/L or higher for each compound) (Richards et al., 2004). Moreover, antibiotics can inhibit the microbiota in WWTPs (Al-Ahmad et al., 1999, Halling-Sørensen, 2001), and hence decrease the performance of a wastewater treatment system. With anaerobic bacteria Sutter et al. (1985) found that 31% out of 362 strains of anaerobic bacteria were susceptible to CIP (tentative breakpoints $< 1.0 \mu\text{g/ml}$), while 58% were resistant ($> 2.0 \mu\text{g/ml}$).

In our study described in Chapter 4, CIP had a significant effect on anaerobic digestion at concentrations of $> 0.5 \text{ mg/L}$ which resulted in a statistically significant reduction in biogas production, and COD removal, and the accumulation of VFAs and SMPs. In addition, CIP also caused a shift in the microbial ecology at 0.5-50 mg CIP/L. The 20% and 50% inhibition concentrations of gas production were 0.11 mg/L, and 4.8 mg/L,

respectively. An increase in the number and concentration of aromatic compounds, with the predominance of p-cresol from 2.5 mg CIP/L, and large quantities of fatty acids in 50 mg CIP/L were also observed. With a reduction in the performance of anaerobic batch reactors when exposed to CIP, it is important to understand how the performance of an AnMBR is affected with CIP present in its feed. Therefore, this chapter will investigate the performance of an AnMBR under long term exposure to CIP. The main goal of this chapter was to evaluate its performance in terms of methane production, COD removal, and the characterization of organic compounds in the effluent under CIP exposure such as VFAs, and low-MW SMPs. Moreover, the long-term impact of CIP exposure to microbial ecology inside AnMBR will also be investigated.

5.2 Experimental and analytical details

The experiment was carried out in the AnMBR with the reactor design and operation as described in Sections 3.1 and 3.2, respectively. During the whole period of operation, the ORP, TMP and pH were monitored and recorded online continuously as described in Section 3.1, and the signal was transmitted to the personal computer. The samples for biogas, COD, VFA, SEC, VFA, low-MW SMPs and TSS were analysed according to Section 3.5.

5.3 Results and discussion

5.3.1 Effect of CIP on the operation of an AnMBR

In the start-up state, phase I, and phase II, the performance of the AnMBR was excellent with 95 ± 2 % (calculated from day 7) and 97 ± 1 % COD removals, respectively (Table 5.1). These COD removal values are consistent with previous research in AnMBRs (Hu and Stuckey, 2006, Luna et al., 2014). Permeate COD was very low, and in the range of 14-24 mg/L, while oxidation reduction potential (ORP) was in the range of -451 ± 6 mV except for some instability when the recycled biogas-bottle was changed. The recycled biogas-bottle was in front of the gas pump to help prevent water entering the gas pump. It should be noted that during operation of the reactor, the changing of this bottle was regularly carried out approximately every 2 months. During the changeover, disturbances in methane composition and biogas volume occurred for a few days afterwards, but this did not disturb either COD removal, or VFA accumulation. Except

for these disturbances, the percentage of methane in the gas was more than 70%, with about 10% carbon dioxide, and this is typical of short retention time anaerobic digesters. Methane gas production in phase II was 247 ± 25 (CH₄)/g of COD removed, and almost no VFA accumulation was observed during these periods.

When CIP was added to the reactor at a concentration of 0.5 mg/L, this had no significant impact on COD removal or biogas production ($p < 0.05$); a stable COD removal of $96 \pm 1\%$ was achieved with effluent CODs as low as 17 ± 6 mg/L. The biogas composition and methane production were constant, and similar to previous Phases; in addition, no VFA accumulation was observed also. When the concentration of CIP was increased to 4.7 mg/L, the system was disturbed after a lag period of 5 days that showed no change in COD removal, VFA and biogas accumulation. Then the COD removal reduced gradually down to 55%, but then recovered slightly after that; the average COD removal over this 91 day period was $78 \pm 13\%$.

Consequently, a lower biogas production, and VFA accumulation in the effluent were observed. The main components of the VFAs were acetate and propionate, and propionate dominated initially, but then after several days the concentration of acetate increased and became dominant. Acetate and propionate were also the main VFAs found in the effluent of the AnMBR at a short HRT (≤ 2 h HRT) (Kunacheva et al., 2017b). However, this was different to the operation of an AnMBR suffering from a pH 11 shock which showed that the major VFAs accumulating were formate, acetate and iso-butyrate (Kunacheva et al., 2017c). In the final phase, no CIP was added, and the AnMBR recovered. During the recovery period VFA accumulation was still observed during the early stages, but this decreased over time, and an increase in methane production and COD removal were also observed. At the end of this period a COD removal of $89 \pm 2\%$ and an effluent containing 55 ± 10 mg COD/L were observed (average of 10 days of operation).

Table 5.1 Overall performance of AnMBR during different operation stages

Phase	COD _{Effluent} (mg/L)	COD removal (%)	Methane yield (m ³ methane/kgCOD removed)	VFAs as COD (mg/L)
Phase I	24 ± 10	95 ± 2	-	< LOD
Phase II	14 ± 5	97 ± 1	247 ± 25	< LOD
Phase III	17 ± 6	96 ± 1	242 ± 26	< LOD
Phase IV	13 ± 7	98 ± 1	245 ± 13	< LOD
Phase V	111 ± 63	78 ± 13	148 ± 65	82 ± 65
Phase VI	68 ± 18	86 ± 4	149 ± 153	44 ± 45

LOD: limit of detection

5.3.2 Effect of CIP on pH and MLSS

After CIP was added to the reactor, a reduction in MLSS and MLVSS was observed after a lag period of 7-10 days; being an antibiotic, CIP has the ability to inhibit the growth of microorganisms. CIP can be classified as “very toxic to aquatic organisms” (EC₅₀ below 1 mg L⁻¹) and “toxic to soil organisms” (Girardi et al., 2011), and the mechanism of CIP in inhibiting bacterial replication is by blocking their DNA replication pathway. During protein synthesis and DNA replication, the unwinding of the double stranded DNA to a single stranded structure is required, and this process is carried out by the enzymes DNA gyrase or DNA topoisomerase. The quinolone antibiotics can bind the A-subunit of the enzyme and block the enzyme, leading to it being unable to replicate or even synthesise proteins, consequently leading to bacteriostasis and cell death finally.

Figure 5.1 Effect of CIP on TSS in the different operational phase in the AnMBR. The CIP was added in phase I to VI with concentrations of 0, 0, 0.5 ± 0.1 , 1.5 ± 0.1 , 4.7 ± 0.7 and 0 mg CIP/L, respectively. Each data point represents the average of three technical replicates while the Error bars represent the standard deviation of the measurements.

The decrease in MLSS and MLVSS in this study indicated the death of microorganisms when exposed to CIP. However, it should be noted that the MLSS and MLVSS only decreased after a short period of increasing over 14 days and 6 days for periods IV and V, respectively. The increase in biomass in phase IV could possibly be due to a lag phase of the process when the CIP had not yet affected growth. However, in phase V the sudden increase in MLSS and MLVSS were not as expected; as during phase III, where a decrease in MLSS was noted, this was expected to decrease or remain constant when the higher concentration of CIP was added in phase IV. Nevertheless, the increase in MLSS and MLVSS at the beginning of phase IV could be due to two reasons. The reason suggested is due to the ability of CIP to inhibit and kill filaments in the sludge (Meng et al., 2012). This controls filamentous bulking, releasing the granular sludge stuck to the reactor wall or membrane of the reactor into the mixed liquor, and consequently increasing the amount of sludge in the mixed liquor (MLSS and MLVSS). After a short

period of increasing MLSS and MLVSS, the growth was inhibited because of antibiotic exposure and the cell concentration decreased after this point.

Figure 5.2 Effect of CIP on the pH in the AnMBR. The CIP was added in phase I to VI with concentrations of 0, 0, 0.5 ± 0.1 , 1.5 ± 0.1 , 4.7 ± 0.7 and 0 mg CIP/L, respectively. Each data point represents the average of pH which is continuously measured (one measurement per second), while the Error bars represent the standard deviation of the measurements.

A slight increase in pH after CIP was added was also observed, as shown in Figure 5.2. This result was in contrast to a study which showed a decrease in pH due to an increase in the VFAs in the anaerobic digester (Aquino and Stuckey, 2004a). In our study, the pH kept increasing even when manual adjustment of the pH to 7 was made by adding HCl 1M once a day during phase V. The reason for this phenomenon can be explained by the ability of antibiotics to kill bacteria through disrupting the proton balance within and outside bacterial cells that was suggested by Bartek (Bartek et al., 2016). In which, antibiotics increased the pH in cytosol while intracellular alkalization were prevented. Thus, a hypothesis here is that CIP also have ability to disrupt the pH homeostasis of bacteria, consequently resulting in an increase in pH inside the AnMBR.

5.3.3 Effect of CIP on size distribution

Figure 5.3 shows the size distribution of the supernatant, the liquid fraction inside the AnMBR. In the literature there have been very few studies on the size distribution of solutes in anaerobic reactors. The molecular weight (MW) distribution of solutes is usually determined by means of SEC (size exclusion chromatography-continuous distribution) or ultrafiltration membranes using stirred cells (discrete distribution) (Barker et al., 1999). The size of the distribution of organic compounds can range from < 1 kDa to > 300 kDa (Barker et al., 2000). In our study we used a SEC method where in the chromatogram the high MW compounds were eluted from the column first and appear at short retention times. In contrast, the smaller MW compounds will take a longer time to elute due to their deeper diffusion into the gel, and hence appear at longer retention times. The organic compounds that adsorb in the RID and UV light at 210 nm, 254 nm and 280 nm will be detected in this study, and these detectors will roughly determine the chemical structure of the organic compounds in solution. The UV measurement at 210 nm is known as the universal wavelength, and can detect almost all compounds, while RID, and UV light at 254 nm and 280 nm are used for the detection of carbohydrates, aromatic compounds and protein-like compounds, respectively.

Overall, the chromatograms of the supernatant before and after CIP addition showed a small difference under low CIP concentration (0.5 mg/L) and a significant difference when high CIP concentration (4.6 mg/L) were applied. The size distribution using SEC of the organic compounds inside the AnMBR showed the large range of size fractions from $>>1522$ kDa to <100 Da. During the start-up conditions in phases I and II, no significant difference in their SEC was observed. However, there was a small change in the MW distribution inside the SAMBR when 0.5 mg CIP/L was added in phase III; the appearance of a peak at around 73 kDa (at 14 min), and an increase in the peak at 8.5 mins. However, after feeding with 4.7 mg CIP/L in phase V, these fractions increased significantly, and in phase VI when no CIP applied, these fractions decreased and then disappeared. The 74 kDa fraction absorbed strongly in the RID detector, but less so at 254 nm, and had almost no absorbance at 280 nm; thus, the fraction that appeared under CIP exposure could have been primarily carbohydrate. The $>>1522$ kDa fraction at 8.5 mins showed strong absorbance in the RID detector and at 254 nm in the UV, so could be a mixture of both aromatics and carbohydrates, or high MW compounds containing both components. Compared with a study on the changing size distribution due to other

stress conditions, such as a change in pH, or a increase in SRT, it was also shown that the high MW fraction increased at both low or high pHs, an increase in SRT, and a decrease in alkalinity (Aquino and Stuckey, 2004a, Kunacheva et al., 2017b, Kuo and Parkin, 1996). Under a pH 5 shock, higher concentrations of carbohydrates (30 k–200 kDa) were found, while higher MW (1500 kDa–0.2 μ m) protein-like compounds were found under the pH 11 shock (Kunacheva et al., 2017b). Kuo and Parkin (1996) observed that the higher MW fraction increased with increasing SRT, so that for an SRT of 40 days up to 76% of the SMP had a MW > 10 kDa. Thus, different operating conditions of an AnMBR could cause an increase in COD, and the organic compounds released were obviously different, which was indicated by the size fraction of the compounds. The compounds generated could be either utilization-associated products (UAP), or biomass-associated products (BAPs) due to hydrolysis and degradation. However, the influent of the AnMBR was comprised only of low MW compounds (< 500 Da), the appearance of high MW compounds after CIP exposure are likely to be BAPs, which are likely to be produced because of cell decay and the turnover of cellular structures. In another study, by determining the DNA/VSS and protein/SMP produced by lysis in an anaerobic chemostat under low alkalinity feed conditions, an increase in high MW compounds also suggested that BAPs were produced as a result of enhanced cell lysis (Aquino and Stuckey, 2004a).

In addition, fewer compounds were found in the effluent compared with the supernatant inside the reactors. In the supernatant, there are many peaks that are >> 1522 kDa which elute in less than 10 min. However, after filtration by the membrane biofilm, all the compounds eluted $\leq$ 10 mins were rejected due to size exclusion and rejection by the membrane and its dynamic fouling layer. In addition, there is little difference in the supernatant before and after adding CIP 0.5 mg/L, and after dynamic membrane filtration, no difference in the effluent was observed. This was caused by membrane rejection as a result of the formation of a gel layer on the membrane, which is agreement with other findings (Jarusutthirak et al., 2002, Shin and Kang, 2003). Nevertheless, the appearance of peaks around 73 kDa when high CIP concentrations were applied (phase V) were still obvious and differ from previous samples with no CIP or low CIP in the feed.

Figure 5.3 Size distribution of organic compounds in the supernatants of AnMBR in phase II (0 mg CIP/L), III (0.5 ± 0.1 mg CIP/L), IV (1.5 ± 0.1 mg CIP/L) and V (4.7 ± 0.7 mg CIP/L). The chromatograms were detected by RID using the SEC method.

5.3.4 Effect of CIP on low molecular weight SMPs

The low-MW SMP compounds identified in this study belonged to various groups, including alkanes, alkenes, esters, alcohols, phenols, N-compounds, fatty acids and S-compounds. Except for fatty acids, the compounds belonging to these groups have been found previously in the literature in the effluent of an AnMBR (Trzcinski and Stuckey, 2010). Hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids, esters, aromatic compounds, N-compounds and others were also found in the headspace of various pure strains of microorganisms (Citron et al., 2012). The bacteria *Chitinophaga* Fx7914 produced more than 200 volatile compounds which included S-compounds, acids, alcohols, ester, and ketones (Nawrath et al., 2010). In addition, aromatic compounds, and N-compounds were also found being produced by strain *Chitinophaga pinensis* DSM 2588 rather than alkanes, cycloalkanes, sulfur-compounds, esters, alcohols, ketones, acids (Citron et al., 2012). However, how and why they are produced is generally not known. Hydrocarbons are biosynthesized by various microorganisms (Albro and Dittmer, 1969, Tornabene and Oro, 1967) in two forms: intracellular hydrocarbons (mainly long-chain C₂₅–C₃₅ n-alkanes), and extracellular hydrocarbons (lower chain lengths) (Ladygina et al., 2006). Extracellular hydrocarbons were believed to have the role of anti-adhesins, and therefore promoted cell aggregation (Nikolaev et al., 2001). These hydrocarbons could be synthesised by two main routes: the “elongation–decarboxylation” pathway, with the addition of a C₂ unit derived from malonyl-CoA following by decarboxylation, and the head-to-head condensation of two fatty acids pathway (Ladygina et al., 2006). The S- compounds are produced through many mechanisms, such as biological reduction, hydrolysis, methylation, and metal catalyzed oxidization (Du and Parker, 2012); the biosynthesis pathway of other compounds was not found in the literature. One of the S-compounds which was sometimes found was Benzenesulfonamide, a type of plasticizer. This compound was found in a batch anaerobic stirred tank reactor (Kuo et al., 1996), in a landfill leachate (Badoil and Benanou, 2009) and in an AnMBR (Trzcinski and Stuckey, 2010), and *Streptomyces* sp. TN262 was proved to have ability to produce this compound (Elleuch et al., 2010).

A change in the total number of groups of SMPs during the operation of the AnMBR with the addition of CIP was observed and shown in Table 5.4; N-compounds were not found in the operation of the AnMBR with no CIP addition. Based on this control, the

number of N-compounds were found to increase up to 13 compounds at the highest CIP concentration (5 mg/L), and then decrease when no CIP was added. Two N-compounds found here (indole, 2-Indolinone) were Indolic compounds that are known to be produced from the metabolism of aromatic amino acids during a protein diet (Nowak and Libudzisz, 2006, Smith and Macfarlane, 1996). Two N-compounds, Benzothiazole and Benzenesulfonamide, were found during an intermediate state only, that is within 24h after adding CIP. Benzothiazole was also found during the intermediate stage when applying 5 mg CIP/L, after that time no benzothiazole was found. In addition, more N-compounds were found when adding 5 mg CIP/L to the feed. Moreover, long chain acids such as 9-Hexadecenoic acid, 4-Methylhexanoic acid and Heptanoic acid were produced in phase VI and phase VII. This differs substantially from the batch tests carried out with anaerobic digestion in our previous experiments, as shown in Chapter 4. In the batch test at a concentration of CIP of 50 mg CIP-HCl/L only, not only were there long-chain fatty acids produced (octanoic acid, nonanoic acid, decanoic acid, etc.), but also phenolic acids such as benzeneacetic acid and hydrocinnamic acid were found.

Figure 5.4 Number of low-MW SMP compounds detected in AnMBR effluent with CIP added in phase III (0.5 ± 0.1 mg CIP/L), phase IV (1.5 ± 0.1 mg CIP/L), phase V (4.7 ± 0.7 mg CIP/L), phase VI (no CIP)

In addition, p-cresol was found when CIP was added, and its concentration increased (up to 49 $\mu\text{g/L}$) when a higher concentration of CIP was added, which is similar to our

findings in batch anaerobic digestion. The phenolic compounds, including p-cresol, were produced from the metabolism of aromatic amino acids in a protein diet, which is similar to Indolic compounds (Nowak and Libudzisz, 2006, Smith and Macfarlane, 1996). However, p-cresol results from tyrosine, whereas indolic compounds are produced from tryptophan (Smith and Macfarlane, 1996).

As discussed in Chapter 4, indole and cresol are known to be carcinogenic (Nowak and Libudzisz, 2006), and the disinfection by-products from SMPs could be more environmentally toxic than the SMPs themselves (Zhang et al., 2015), and are often genotoxic, mutagenic and/or carcinogenic, with obvious adverse effects on human health (Richardson et al., 2007). Furthermore, more N-compounds were found which could be more harmful as N-containing disinfection by-products are more toxic, and up to 140 times more than carbon-containing disinfection by-products (Plewa et al., 2007). Thus, the higher concentrations of SMPs when treating antibiotics, such as Indole, p-cresol, and other phenolic compounds, and also N-compounds could cause potential adverse effects to the ecosystem and human health.

5.4 Conclusions

Overall, the presence of antibiotics in the reactor feed appear to have had more effects than simply acting as growth inhibitors on AnMBR performance which is manifest in reduced biogas production and COD removal. CIP appears to influence the composition of the organic compounds produced in term of VFA accumulation, size distribution and low-MW SMP accumulation. Although at low CIP concentrations of 0.5 mg/L their effect was not obvious on COD removal, VFA accumulation and methane yield, it can be seen in its effects on pH, SMPs and TSS/VSS. A significant effect on all the parameters tested was seen at high concentrations of CIP exposure (5 mg/L), including COD removal, VFA accumulation, biogas production as well as SMP production. The analysis of low-MW SMPs showed an increase in the number of various groups of compounds such as esters, N-compounds, acids, and an increase in the concentration of phenol compounds (p-cresol) when the AnMBR was operated with CIP in the feed.

CHAPTER 6

Fate and removal of CIP in an AnMBR

Outline:

6.1 Introduction

6.2 Material and methods

6.3 Results and discussion

6.4 Conclusions

6.1 Introduction

The removal of CIP in WWTPs has been shown to occur in the literature with the use of conventional activated sludge, and a few studies using MBRs. However, the performance of these WWTPs using biological treatment was highly variable, with removal efficiencies ranging from zero to nearly complete removal (Michael et al., 2013). In addition, the studies available in the literature often do not provide enough detail on the fate and transport of antibiotics, including CIP, during the wastewater treatment process (Michael et al., 2013). It was shown that SRT is an important reactor parameter in the degradation of antibiotics as high SRTs will enrich for slow-growing bacteria, providing a greater diversity in the microbiome, and thus the ability of the culture to degrade antibiotics. A membrane associated with the reactor will also provide a high SRT by eliminating the washout of biomass, and therefore a higher performance is obtained. Some studies used membrane bioreactors to treat wastewater containing CIP; in an aerobic MBR the CIP removal was between 54-93% when treating hospital wastewater, where a hollow-fibre MBR showed a lower removal efficiency compared with a flat-sheet MBR (Nguyen et al., 2017). The reason for these differences was the higher sludge concentration in the flat-sheet MBR compared to the hollow fibre MBR (Nguyen et al., 2017), however, the mechanism of CIP removal was not investigated. CIP removal in a two-stage MBR that consisted of an anoxic reactor and an aerobic MBR reactor connected in series was 58% (Hamjinda et al., 2017). The addition of one more stage with the bioaugmentation of *Pseudomonas putida* immobilized in polyvinyl alcohol and sodium alginate beads was found to increase the CIP removal to 90%

(Hamjinda et al., 2017). In addition, more CIP transformation products were detected in the anoxic tank than in the entrapped *P. putida* reactors and MBR tanks (Hamjinda et al., 2017). Furthermore, CIP was shown to be biodegraded in several systems such as in saline sewage systems, under aerobic and anoxic conditions, and in a drinking water biofilter (Dorival-Garcia et al., 2013, Hamjinda et al., Li and Zhang, 2010, Liao et al., 2016, Liu et al., 2013a, Liu et al., 2013c), whereas it was found to be resistant in other systems, including freshwater sewage systems, methanogenic cultures and digested sludge (Golet et al., 2003, Li and Zhang, 2010, Liu et al., 2013c). Currently, there has only been one bacterial strain, *Labrys portucalensis*, that has been documented to degrade CIP (Amorim et al., 2014). The study suggested two different metabolic pathways of CIP, which included the formation of $C_{17}H_{20}N_3O_4$ and $C_{15}H_{17}FN_3O_3$ by the defluorination and oxidation of the amine moiety.

However, the effectiveness of removing CIP in an AnMBR has not been investigated, although the AnMBR has many advantages in wastewater treatment, and is a potential reactor design for future application. Therefore, the main goal of this present work was to evaluate the removal of CIP by an AnMBR at a high but environmental consistent concentration of CIP. Besides investigating removal efficiency, the major routes for CIP removal will be investigated to see whether CIP is biodegraded in an AnMBR, and by what mechanisms CIP is removed.

6.2 Experimental and analytical details

An AnMBR was used with the same configuration and operational details as described in part 3.1 and 3.2, respectively. CIP was added on day 86 from start-up, and in the first period of 44 days, an average of 0.5 ± 0.1 mg/L was added to the feed. After this, for a short period of 11 days a feed with 1.5 ± 0.1 mg/L was applied before finally feeding with a higher concentration of 4.7 ± 0.7 mg/L for 93 days. The purpose of the intermediate period of adding 1.5 mg/L was to prevent too steep a shock load that may have occurred if the feed had gone from 0.5 mg/L to 4.7 mg/L. Finally, the reactor was operated without CIP for 45 days after CIP addition to determine the rate of desorption of CIP, as well as the biological recovery of the AnMBR after its exposure to the antibiotic.

The biosorption properties of CIP onto AnMBR sludges were investigated by using the batch experiments (both kinetic and isotherm) as described in parts 3.3.2 and 3.3.3. The measurement of CIP and scan of CIP intermediate were as documented in part 3.5.10 and 3.5.11, respectively. The fate of CIP removal was based on the mass balance of CIP as described in part 3.6.

6.3 Results and discussion

6.3.1 CIP removal in AnMBR

The performance of the AnMBR was as documented in Chapter 5. In brief, COD removal of the AnMBR was excellent with $> 95\%$ COD removal during the start-up period, and during the addition of a low concentration of CIP (0.5 mg/L and 1.5 mg/L) (Fig. 6.1.a). As result of this high COD removal efficiency, the permeate in those periods had a low COD concentration, and was in the range of 14 -24 mg COD/L. However, when the concentration of CIP was increased to 4.7 mg/L, the performance decreased, with an average COD removal of $78 \pm 13 \%$. In addition, VFAs and biogas production and other parameters were affected. In the final phase with no CIP added, the COD removal increased gradually, and reached around $89 \pm 2 \%$ on average over the last seven days of the experiment.

The CIP removal efficiency in the AnMBR was monitored over 120 days after CIP was added to the system, and this is shown in Fig. 6.1.b. For the first 40 days, CIP was fed at a concentration 0.5 ± 0.1 mg/L, and an average CIP removal of $50.4 \pm 17.6\%$ was achieved. A high removal ($76.4 \pm 3.9\%$) of CIP was also observed when a higher CIP concentration was applied of 1.5 ± 0.1 mg/L. This value was quite comparable with the removal efficiency of a hollow-fibre MBR with 54-70% removal efficiency (Nguyen et al., 2017), and a two stage MBR that consisted of an anoxic reactor and an aerobic MBR reactor connected in series, with 58% CIP removal (Hamjinda et al., 2017). However, the flat-sheet MBR showed a higher CIP removal with 76-93% reduction (Nguyen et al., 2017), and this was explained by the higher sludge concentration (Nguyen et al., 2017). Moreover, CIP removal could be enhanced by bioaugmenting with *Pseudomonas putida* in polyvinyl alcohol and sodium alginate beads (Hamjinda et al., 2017). However, theoretically there is a potential of increasing CIP removal efficiency in a two stage MBR, but the effect of adding this stage to an AnMBR is questionable.

When a higher CIP concentration was added to the reactor, the CIP removal gradually reduced, and flattened out after 20 days, with a removal efficiency $< 20\%$. The lower CIP removal efficiency occurred at the same time as a lower COD removal, and the saturation of CIP adsorbed onto the biomass. This suggested that the route for CIP removal could either depend on biodegradation, or adsorption, and this question will be examined in the next section. In the last period, where no CIP was added into the feed, CIP was still detected in the effluent due to the desorption of CIP from the sludge into the liquid phase. However, the concentration of CIP gradually decreased and was eventually lower than its detection limit.

Figure 6.1 Performance of AnMBR in terms of; a) COD removal, b) CIP removal efficiency, and, c) CIP adsorbed onto the sludge. In phase III to VI, 0.5 ± 0.1 , 1.5 ± 0.1 , 4.7 ± 0.7 mg CIP/L and 0 mg CIP/L was added, respectively. Each data point represents the average of three replicates, while the Error bars represent the standard deviation of each measurement.

Besides the monitoring of CIP in the effluent and feed, the amount of CIP absorbed/adsorbed onto biomass was also measured (Fig. 6.1.c). This data showed the slow and gradual increase in the amount of CIP on the biomass during the application of 0.5 mg CIP/L. When the concentration of CIP increased, the amount of CIP on the biomass also increased, and became saturated at approximately 2 mg/g. The amount of CIP sorbed onto the sludge was in the range found in the past, with CIP on anaerobic digested sludge ranging from 0.5-4.8 mg/kg dry weight (Lindberg et al., 2005).

6.3.2 Fate of CIP removed in an AnMBR

From the concentration of CIP in the influent, effluent, and sludge inside the reactor, and wasted from the reactor, a mass balance was carried out to determine the mechanism of CIP removal inside the AnMBR. The chemical-physical properties of CIP with a low Henry's Law constant of 5.09×10^{-19} (atm·m³/mol), a significant molecular weight (331.35), and the presence of two ionizable functional groups (a carboxylic and piperazinyl group) suggests that CIP was stable from hydrolysis/photolysis and was not easily volatilised. When operating an AnMBR with a similar configuration without anaerobic sludge for 30 h feeding similar CIP concentrations, the removal by volatilization, hydrolysis or sorption to the feed tubing or Plexiglass reactor walls was statistically zero. In addition, membrane filtration resulted in no rejection of CIP since the size and molecular length of CIP were much smaller than the membrane pores (effective pore size of 0.2 µm). Even with biofiltration, the rejection of CIP was zero, as no statistical difference occurred between the supernatant inside the AnMBR, and effluent samples during the operation of AnMBR. Therefore, the mechanism of CIP elimination in a biological AnMBR was mainly from sorption and biodegradation/biotransformation, discharge with the effluent and discharge with waste sludge (Fig. 6.2).

In the initial stage of feeding CIP at 0.5 mg/L, biological degradation/transformation and discharge through the effluent were the two main ways of removing CIP from the AnMBR. However, removal by degradation/transformation made a considerably higher contribution to the overall removal. The removal by adsorption was low (5%) and decreased over time in this period. When the CIP concentration in the feed was increased, the contribution of adsorption to the overall removal increased due to an increase in the amount of CIP sorbed to the sludge. The maximum contribution of

sorption to sludge could contribute up to 26% of the total removal; however, this contribution decreased when the sorbed amount of CIP to the sludge was saturated. This finding is in contrast to some findings in a WWTP which showed that sorption was important in removing CIP from wastewater (Castiglioni et al., 2006). The reason for this discrepancy could be due to the lower concentration in the WWTP, and a lower biomass concentration as this shows that biomass concentration plays an important role in CIP removal (Dorival-Garcia et al., 2013). CIP sorption to sludge can either be by the bioaccumulation of solute into lipids in the cell, which depends on hydrophobicity, or the electrostatic interactions between the amino groups in CIP and the cell surface of the microorganisms. However, CIP is a hydrophilic compound with a low K_{ow} , which would be poorly absorbed onto/into sludge; therefore, adsorption by electrostatics seems to be the most credible mechanism in this case. CIP is readily adsorbed to sludge via electrostatic interactions between the positive charge of the amino groups in CIP, and the negatively charged cell surface of micro-organisms (Ternes et al., 2004). Biotransformation was the dominant mechanism of removal of CIP in the initial stage; however, right after the AnMBR performance was disturbed by the high concentration of CIP, removal by biological degradation/transformation reduced significantly. At the same time, most of CIP was not removed but discharged through the reactor effluent.

In the section below, more detail on the two major ways of removing CIP, including adsorption and biodegradation, were shown.

Figure 6.2 Fate of CIP removal in the AnMBR during the addition of CIP in phase III (0.5 ± 0.1 mg CIP/L), phase IV (1.5 ± 0.1 mg CIP/L) and phase V (4.7 ± 0.7 mg CIP/L)

6.3.3 Biodegradation of CIP

The biodegradation of CIP has been observed in several biological systems, such as in saline sewage systems, while no biodegradation was observed in freshwater sewage systems (Li and Zhang, 2010). This was due to the different microbial communities that develop in the different environments, and that the special fluoroquinolones degrading microorganisms existed in the saline activated sludge only, but not in the freshwater sewage system (Li and Zhang, 2010). Several possible degradation routes of CIP by the brown rot fungus *G. striatum* were suggested, and include oxidative decarboxylation, defluorination, hydroxylation at C-8, or oxidation of the amine moiety (Wetzstein et al., 1999). Twenty-four CIP transformation products were detected in the two-stage MBR, with more products in the anoxic tank than in the reactors with entrapped *P. putida* cells, and MBR tanks (Hamjinda et al., 2017). Under aerobic and anoxic conditions, and in drinking water biofilters, the biotransformation of CIP was reported (Dorival-Garcia et al., 2013, Hamjinda et al., Liao et al., 2016, Liu et al., 2013a, Liu et al., 2013c), and Liao et al. (2016) reported that CIP could act as a sole carbon and nitrogen source. It was suggested that CIP could be utilized as a sole carbon and nitrogen source which could be utilized by both the original microbiota, and acclimated microbiota originating from a drinking water biofilter (Liao et al., 2016). However, there was only one bacterial strain (*Labrys portucalensis*) that has been documented to degrade CIP (Amorim et al., 2014), although the presence of the genera *Pseudoxanthomonas*, *Stenotrophomonas*, *Phenylobacterium*, and *Leucobacter* may play important roles in CIP removal (Liao et al., 2016). However, there was no significant removal of CIP observed under methanogenic conditions in a field evaluation (Golet et al., 2003), and in a mixed methanogenic culture and digested sludge, no CIP biodegradation was observed (Liu et al., 2013c).

In our study, a few intermediate compounds were found in the effluent when a high concentration of CIP was applied, including intermediates with molecular weights of 305 and 329 obtained by LC-MS/MS, and 3,4-Dimethyl-2(1H)-quinolinone obtained by GC-MS (Fig. 6.3). The 305 (C1) and 329 (C2) derivatives are produced during the defluorination and oxidation of the amine moiety, respectively, and they have been documented in a number of past studies (Amorim et al., 2014, Turiel et al., 2005). However, 3,4-Dimethyl-2(1H)-quinolinone has not been reported in any study since

most of intermediate compounds identified were found using LC-MS/MS, but not GC-MS for the more volatile intermediate compounds. The C2 derivative (MW 329) degradation product shows several fragments at an m/z of $(M+H)-18$, -44 , -58 , and the C1 derivative (MW 305) degradation product results in fragments at an m/z of $(M+H)-17$, -43 , -75 . In the MS spectrum of the C2 derivative, the first fraction with a loss of 18 Dalton corresponds to the loss of a H_2O molecular from the carboxylic group. The loss of the 44 and 58 fractions are the fragments of the piperazinyl ring of the fluoroquinolones. In the C1 degradation products, the opening of the ring happens as the result of a substitution in the piperazinilic group; hence, the -17 and -43 fractions were obtained from the amino chain. It can also be seen that the concentration of these intermediate compounds was very low, and that no intermediate compounds were found in the 0.5 mg CIP feed, and that only approximate 1 $\mu\text{g/L}$ for 3,4-Dimethyl-2(1H)-quinolinone was quantified when the feed contained 5 mg CIP/L. Furthermore, the abundance of the C2 and C1 derivatives was much lower compared to CIP (the peak abundance was less than 1% compared to CIP). Hence, the route to CIP removal was through biodegradation rather than biotransformation to other intermediates.

Figure 6.3 The potential intermediates for CIP biodegradation/transformation

6.3.4 Sorption of CIP onto biomass

6.3.4.1 Effect of contact time on adsorption

The sorption of CIP onto anaerobic sludge in the AnMBR reactor that has never been exposed to CIP in the batch study was investigated, and the CIP adsorption kinetics are shown in Fig. 6.4.a. The results show that the adsorption onto biomass was very fast initially, within 10 mins (2 mins mixing + 8 mins analysis process). After this time the adsorption rate decreased until equilibrium was reached. This behaviour is normal for adsorption and can be explained by the availability of adsorption sites on the adsorbent initially, and as the adsorption sites are occupied by the adsorbate, the adsorption rate decreases and then reaches equilibrium. The adsorption kinetics of CIP was analysed using either a Pseudo-first or second order model (Fig. 6.4.b, c), and the constants and correlation coefficients based on the kinetic models are shown in Table 6.1. It was found that the correlation coefficients for the Pseudo-second-order model (both $R^2 = 0.99$) were much higher than the values obtained from the Pseudo-first-order model ($R^2=0.59$), suggesting that the adsorption mechanism of CIP onto biomass followed a pseudo-second order kinetic mechanism. The values for the rate constant and amount of CIP adsorbed onto the sludge were 0.18 g/mg/min and 1.10 mg/g, respectively. As no values for the rate constant of CIP adsorption onto anaerobic sludge were found in the literature, no comparison could be made. However, when compared to the values of the adsorption onto Schorl, the most common form of Tourmaline mineral, the adsorption rate constant was lower, ranging from 0.05-0.1 g/mg/min, whereas the amount of CIP adsorbed onto the sludge was higher at 5.7-7.0 mg/g (Yin et al., 2017).

Table 6.1 Kinetic parameters and correlation coefficients based on the pseudo-first and pseudo-second order model

Pseudo-first order		Pseudo-Second order	
q_e (mg/g)	1.09	q_e (mg/g)	1.10
k_1 (1/min)	0.01	k_2 (g/mg/min)	0.18
R^2	0.59	R^2	0.99

Figure 6.4 Non-linear (a), Pseudo-first order (b), and Pseudo-second order (c) model fit of CIP adsorption onto AnMBR sludge.

6.3.4.2 Adsorption isotherm

The isotherm results were analysed using the Freundlich and Langmuir models, as shown in Fig. 6.5. It was found that the sorption of CIP could be effectively described by both the Langmuir and Freundlich isotherms, with correlation coefficients (R^2) of 0.94 for both models. The Langmuir isotherm describes the adsorption at specific homogeneous sites within the adsorbent, whereas the Freundlich isotherm describes the non-uniform distribution of adsorption on a heterogeneous surface. The calculated distribution coefficient for adsorption of CIP onto anaerobic sludge was calculated based on the parameters in the Freundlich model, which showed a $K_d = 3.5$ (L/g). The value was in the range of CIP adsorbed onto freshwater sewage sludge, 2.3-5.1 L/g (Li and Zhang, 2010), and higher than the values in other types of sludge, including aerobically digested biosolids, saline sewage sludge and aerobic culture biomass, with K_d s ranging from 0.3-2.8 L/g (Li and Zhang, 2010, Liu et al., 2013b, Wu et al., 2009). Furthermore, the Freundlich constant n was equal to 1.3, lying in the range of 1 to 10, indicating that CIP sorption onto the bio-sorbent was favourable. In addition, the maximum adsorption capacity found from the Langmuir model was equal to 11.5 mg/g in our study, and this was lower than that found with anaerobic sludge in another study of 37.9 mg/g (Liu et al., 2013a). Our value was lower presumably due to the difference in initial concentrations of CIP; in our study the CIP concentration used was only 5 mg/L, while it was 100 mg/L in the study cited in the literature.

Table 6.2 Constants and correlation coefficients of adsorption isotherms for the adsorption of CIP onto anaerobic sludge.

Langmuir equation		Freundlich equation	
q_m (mg/g)	11.53	K_f ($\text{mg}^{1-n} \cdot \text{L}^n/\text{g}$)	2.42
b (L/mg)	2.95	n	1.29
R^2	0.94	R^2	0.94

Figure 6.5 Langmuir (a) and Freundlich (b) adsorption isotherms fit of CIP adsorption onto AnMBR sludge

6.4 Conclusions

The removal of the antibiotic CIP in an AnMBR was examined in a long-term operation which showed that a 50-76% CIP removal was possible when CIP was fed at a concentration of 0.5-1.5 mg/L. However, at a higher CIP concentration, 4.7 mg/L, the removal of CIP was seriously affected, and the removal efficiency decreased to < 20%. Biological degradation was the main mechanism for removing CIP when the feed contained < 1.5 mg/L, although a large fraction of the CIP was not degraded and was discharged in the effluent. The adsorption of CIP onto the sludge contributed to only a small fraction of the total removal, and an even smaller portion of the CIP removal was due to the amount adsorbed to the waste sludge discharged. CIP was biodegraded to some degree in the AnMBR, with a few intermediate compounds detected using

sophisticated analytical techniques. However, with higher CIP concentrations in the feed, most of the CIP was not removed, but discharged in the effluent.

CHAPTER 7

Post-treatment of AnMBR effluent by activated carbon³

Outline:

7.1 Introduction

7.2 Material and methods

7.3 Results and discussion

7.4 Conclusions

7.1 Introduction

As shown in Chapter 5, the AnMBR resulted in good performance for wastewater treatment, even with CIP in the feed. At the high concentration of CIP (4.7 mg/L), the performance of the AnMBR was considerably reduced, with COD removals reduced from > 95% to $78 \pm 13\%$, corresponding with a COD in the effluent of 111 ± 63 mg COD/L. The effluent during this period did not satisfy the discharge regulations of several regions such as Singapore, Japan and European, with their discharge regulations ranging from 60 to 125 mg COD/L depending on the country and class of waters being discharged into (Japan, 2015, Legislation, 1994, Singapore, 2017). The MW distribution of the effluent showed that SMPs had a wide range of MWs. Characterization of anaerobic effluents showed the existence of a variety of organic compounds, including VFAs and low-MW SMPs which are released during metabolism and the biomass decay of microorganisms, and identification of the low-MW SMPs revealed that most of the compounds were long chain alkenes, alkanes, acids, esters and aromatic compounds. Noticeably, p-cresol and a few N-compounds were analysed and found to be at high concentrations when exposed to CIP, especially p-cresol which was found at concentrations up to 49 µg/L in the effluent, and some phenolic and indolic compounds

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have been shown to be carcinogenic (Nowak and Libudzisz, 2006). Besides this increase in SMPs, a high concentration of CIP was not removed in the AnMBR and still appeared in the effluent. Being an antibiotic, the incomplete treatment and presence of CIP in the AnMBR effluent may cause potentially adverse effects to the ecosystem and human health if they are discharged to the environment, as discussed previously. Disinfection by-products can also be produced from SMPs, and some of those products are genotoxic, mutagenic and/or carcinogenic, with obvious adverse effects on human health (Richardson et al., 2007). Moreover, disinfection by-products produced from N-compounds, which were produced in significant concentrations under CIP exposure in our previous experiment (Chapter 4), are more toxic than carbon-containing disinfection by-products (Plewa et al., 2007).

Hence, post-treatment of AnMBR effluents is needed to satisfy the discharge and recycling requirements, as well as to remove potentially toxic compounds such as phenol and N-compounds. This is very important in the interest of recycling of wastewater, and this part of WWT has been increasing due to the lack of fresh water around the world. A variety of methods have been evaluated in the literature to remove organic carbon from AnMBR effluent. Adsorption by activated carbon was found to be one of the most efficient treatment methods (Trzcinski et al., 2011, Vyrides et al., 2010), and PAC and GAC can remove up to 84% and 80% COD, respectively (Trzcinski et al., 2011). Adsorption by activated carbons is also an effective and well-known process for water treatment, however, activated carbon adsorption does not remove all organic compounds, and Gur-Reznik et al. (2008) showed about 24 % of the dissolved organic matter was non-adsorbable, mostly being hydrophilic and transphilic compounds. Also, high MW organic compounds present in anaerobic effluents were found to be more adsorbable than low MW compounds (Barker et al., 1999, Trzcinski et al., 2011, Vyrides et al., 2010). The < 1 kDa fraction was only removed by 70% by PAC, while higher MW fractions had a higher removal efficiency at 90-100% (Vyrides et al., 2010). However, up to now the exact identity of these compounds which are removed, or remain after adsorption, is limited due to the inability of the existing analytical methods to identify these solutes specifically.

Therefore, the aim of this study was to investigate the removal of organic compounds in AnMBR effluents treating synthetic wastewater containing CIP using different

activated carbons. The objective of this study was to compare and characterize the composition of treated effluent, and the size distribution and removal of specific compounds, including VFA and low-MW (< 580 Da) SMPs, was focused on.

7.2. Experimental and analytical details

The AnMBR effluent samples were taken during the operation of the reactor in phases II, IV, VI under the operational conditions and performance shown in Chapters 5 and 6. In phase V, two effluent samples were taken on days 15 and 30 from the beginning of the phase. Two effluent samples were taken in this period due to the significant difference in the VFA and SMP composition and concentration analysed in this period. Effluent samples were denoted as EFF-I, EFF-II, EFF-III and EFF-IV corresponding with effluent from phases II, V, and 2 effluent samples in phase VI, respectively; these denotations will be used for the rest of this study. No effluent samples were taken in phase I, III and IV as the effluent from phases I and IV were shown to be similar in characteristics in terms of COD concentration, SMP composition, VFA accumulation, and SEC size distribution, and these are shown in Chapter 5. Despite this, in phase VI where the AnMBR was operated after long periods of high CIP exposure at 4.7 mg/L, a significant difference in the initial and later periods was observed, including VFA accumulation, COD concentration, and especially SMPs accumulation. Therefore, two samples in the initial and later periods of phase VI were taken for testing; in brief, four effluent samples with COD concentrations of 9, 238, 56 and 24 mg/L, respectively. EFF-I and EFF-IV contained no VFAs, whereas EFF-II contained 162 and 81 mg/L and EFF-III contained 29 and 5 mg/L of acetic acid and propionic acid, respectively.

Six types of activated carbon were used in the adsorption experiment with their properties listed in Table 3.3. The experiment was as described in part 3.3.4. All the effluent samples were filtered through 0.45 µm filters for COD and SMP analysis, and further filtration through a 0.2 µm filter was carried out for VFA, CIP and SEC analysis. The characterisation of size distribution (SEC), low-MW SMP and analysis of COD, VFAs, CIP and SMP for the AnMBR effluent have been described in part 3.5. The mass

balance of COD for the contribution of VFA and SMP compounds to total COD concentration was carried out following part 3.7.

7.3. Results and discussion

7.3.1 COD removal

A kinetic test on one activated carbon, SA2, was carried out to roughly determine the adsorption behaviour of activated carbons over time. The kinetics of SA2 showed that most of the adsorption was fast, within 1 h, and then after this the adsorption rate was a lot lower (Fig. 7.1.a). A Pseudo first order and Pseudo second order model were used to model the kinetic adsorption behaviour, and these resulted in correlation coefficients, R^2 , equal to 0.59 and 0.99, respectively. Therefore, the kinetic adsorption behaviour which showed the best fit was the Pseudo second order (Fig. 7.1.b) model, which means that the rate of adsorption was second order and depended on both the concentration of COD in the effluent, and the amount of activated carbon. Fast adsorption in the initial stages, with slower adsorption after a certain time, was revealed by the adsorption of high MW solutes onto activated carbon, which can block a significant fraction of the pores, resulting in slower adsorption rates of lower MW compounds (Vyrides et al., 2010). From this initial kinetic study, the equilibrium time for the adsorption experiments carried out in this study was set at 24 h to ensure that the low MW-compounds had time to adsorb.

Figure 7.1 Kinetic study of activated carbon SA2 (dose 2 g/L, shaking speed 150 rpm, room temperature 25°C, n = 3): (a) COD removal versus time; (b) Pseudo-second order kinetics

As described previously, the effluent samples used in this study had different COD concentrations of 9, 238, 56, and 24 mg/L corresponding to EFF-I, EFF-II, EFF-III and EFF-IV, respectively. The adsorption of activated carbon at a dose of 1 g activated carbon/L showed that the removal efficiency ranged from 22 to 82% (Fig. 7.2) for EFF-II, EFF-III and EFF-IV. In EFF-I, no COD removal could be measured since the treated effluent had a COD concentration lower than the detection limit (< 5 mg COD/L). In a study using PAC to treat AnMBR saline effluent, a reduction of 80% in the dissolved organic carbon was observed at a PAC dose of 1.7 g PAC/L (Vyrides et al., 2010). This value was lower compared with an aerobic MBR effluent, where 80%– 90% organic removal was achieved using GAC (Gur-Reznik et al., 2008). Comparing the characterization of the activated carbons (BET, pore volume and pore size) (Table 3.3), no direct correlation between COD removal and these parameters could be seen.

However, with the group of PACs only, this showed that the PACs with a higher BET resulted in better performance compared with those PACs with a lower BET surface area. Two GACs, FD and NRS, resulted in a higher COD removal than the other PACs in EFF-III; this was unexpected as generally GACs result in a lower performance than PACs. However, this data seems reasonable as adsorption could depend on many factors such as area, surface functional group, pore size distribution, size of the activated carbon, etc. In addition, the physicochemical properties of the effluent such as its initial concentration, type and MW distribution, and chemical structure of the organic compounds in the effluent would also affect the adsorption capacity of the ACs. The next section analyses the SMP size distribution, and the removal of VFAs, and SMPs, and together may explain the overall COD removal.

7.3.2 Molecular weight distribution

The size distribution of organic compounds in the AnMBR effluents showed a wide range of MWs, with a range from < 0.1 kDa to 1,522 kDa, which was in a similar range to that found in other studies (Aquino et al., 2006, Aquino and Stuckey, 2002, Barker et al., 1999). Figure 7.3 showed the size distribution of the effluent and treated effluent using different detectors. Comparing the abundance of peaks from different detectors can roughly determine the properties of specific organic compounds, and hence the RID detects the appearance of carbohydrates, while UV at 254 and 280 nm identifies aromatic and protein like compounds, respectively, whereas UV at 210 shows almost all types of organic compounds.

The size distribution of the AnMBR effluents taken for the experiment varied considerably, although there were always three fractions present of $> 1,522$ kDa, 134 kDa, and < 0.1 kDa. For these fractions, the $> 1,522$ kDa fraction was generally low in EFF-I, EFF-III and EFF-IV, but was more concentrated in EFF-II. This difference could also be seen in the appearance of the > 0.6 kDa fraction in EFF-II (Fig. 7.3) and the > 72.8 kDa fraction in EFF-III as well (Fig. 7.4).

After adsorption by activated carbon, some fractions showed a very high removal efficiency, while others showed little or no removal; a summary of the removal efficiencies of the major fractions based on a reduction in peak areas is shown in Table 7.1. From this detailed analysis, the fractions at 134 kDa and < 0.1 kDa were difficult to remove, with no reduction or very low removal efficiency ($< 6\%$) from EFF-I, EFF-III

and EFF-IV. The low MW fraction (<1 kDa) has also been identified as the most difficult fraction to adsorb onto PAC (Vyrides et al., 2010). Furthermore, the < 0.1 kDa fraction is most likely carbohydrates as it was identified with the RID at a high intensity, whereas with the other detectors there was a very low or no signal. This low MW fraction has a high solubility in water, and contained VFAs and low MW compounds which are difficult to remove (Stenzel, 1993). The removal efficiency based on the ACs used was; FD < NRS < HYD $\approx$ SA2 $\approx$ WP $\approx$ SAS generally. The trend depended strongly on particle size and BET surface area, and the two GACs (FD and NRS) with the largest particle size (mm) had lower adsorption capacities than the PACs, even though FD and NRS had a greater BET surface area than HYD, SA2 and WP. The effect of particle size on the adsorption of organics from an AnMBR effluent was found in a past study that showed that activated carbon with small particles (≤ 0.25 mm) adsorb better than larger particles (≤ 0.75 mm) due to their higher diffusional transfer rate (Vyrides et al., 2010). In addition, the 134 kDa fraction was also difficult to remove, with a removal efficiency ranging from 17-68%. This fraction was found by almost all the detectors, so it could be a mixture of many polymeric compounds, including aromatics, proteins and carbohydrates. The complexes in this fraction may also be hard to adsorb completely, and thus reduce the removal efficiency of this fraction.

Figure 7.2 COD removal by activated carbons from an AnMBR effluent. EFF-II, EFF-III and EFF-IV contain 238, 56 and 24 mg COD/L, respectively. Each bar represents the average of three replicates, while the Error bars represent the standard deviation of the measurements.

Despite the above considerations, the high MW compounds of around 1522 kDa, 73 kDa and 0.6 kDa all decreased significantly, which were primarily aromatic compounds (as they showed up in the peaks at UV 254 nm). The reduction of these fractions was greater than 85%, and ranged up to 100%, except for the use of WP to remove the 1522 kDa fraction. WP showed the lowest removal efficiency, and this could be due to the smaller pore volume (0.1 cc/g) of this PAC compared with other PACs (0.28, 0.26 and 0.33 cc/g for HYD, SA2 and SAS respectively).

Vyrides et al. (2010) showed that the fractions of high MW compounds were more adsorbable than low MW compounds due to the lower water solubility (hydrophobicity) of the high MW compounds. However, this is not always necessarily true, as in this case the high MW fraction, 134 kDa, had a lower removal efficiency than the low MW fraction, 72.8 kDa and 0.6 kDa. Our findings suggest that the size of the organic compounds is not the key to determining the adsorption efficiency, but the chemistry of these fractions. Hence, if the 1522 kDa, 72.8 kDa, and 0.6 kDa fractions have similar chemical properties and are aromatic compounds, then they are easy to remove by activated carbon. Whereas, the 134 kDa fraction was more difficult as it is more complex, and could be a mixture of many components, and the < 0.1 kDa fraction contains VFAs and low MW compounds that are very soluble in water, and so difficult to adsorb.

Table 7.1 Summary of the removal efficiency (%) of some major MW fractions.

Fraction	Effluent type	Types of activated carbon					
		FD	NRS	HYD	SA2	WP	SAS
1522 kDa	EFF-II	85	93	91	89	52	97
134 kDa	EFF-I	17	35	55	62	56	59
	EFF-II	20	51	54	53	52	61
	EFF-III	28	35	68	66	62	53
	EFF-IV	35	46	54	53	61	50
72.8 kDa	EFF-III	100	100	100	100	100	100
0.6 kDa	EFF-II	89	100	94	100	100	100
< 0.1 kDa	EFF-I	0	0	0	0	0	0
	EFF-II	20	75	55	88	95	94
	EFF-III	0	4	1	6	6	4
	EFF-IV	0	0	0	0	0	0

Note: the reductions were calculated based on the peak area of each fraction that appears in the UV 210 nm detector for all cases, and the RID detector for the < 0.1 kDa fraction.

Figure 7.3 Size distribution of organic compounds in AnMBR effluent (EFF-II) and effluent treated by activated carbons using SEC method. Detectors used were RID (a), PDA detector at wave length of 210 nm (b), PDA detector at wave length of 254 nm (c), PDA detector at wave length of 280 nm (d).

Figure 7.4 Size distribution of the AnMBR effluent and treated effluent: (a) EFF-I, (b) EFF-III, (c) EFF-IV using a PDA detector at a wavelength of 210 nm.

7.3.3 VFA removal

The VFA content in the AnMBR, as well as other anaerobic treatment processes, can fluctuate substantially under stress conditions; however, no VFAs are normally detected during steady state, although VFAs can be very high during disturbances and stress (Aquino and Stuckey, 2003, Kunacheva et al., 2017b). In our study, two effluents, EFF-II and EFF-III contained VFAs because high concentrations of CIP were being added, while the other two effluent samples did not. After adsorption by activated carbon, the removal efficiency for acetate and propionate ranged from 13-100% and 39-100%, respectively, for all the activated carbons tested on EFF-III (Fig. 7.5). However, the removal of VFAs from EFF-II was very low, ranging from 2-22% removal for acetate, and 0-11% for propionate. In the literature, the adsorption capacity of VFAs was shown to be different for different VFAs, with a maximum capacity based on the Langmuir model, $q_{\max}$, of 5.80 mg/g for acetate, and 0.1 mg/g for propionate (Baeta et al., 2013).

Two GACs, FD and HYD, showed noticeably better performance than PACs with VFA adsorption, especially with EFF-III. This might be explained by the mechanism of adsorption which involves intraparticle diffusion consisting of pore-volume diffusion, and surface diffusion. Pore volume diffusion involves the diffusion of an adsorbate inside the micro and meso-pores of the adsorbent, while surface diffusion involves the diffusion of an adsorbate to the surface of the adsorbent. Small molecules have high surface diffusion, which means that these molecules can diffuse quickly into the pore volumes (Ocampo-Pérez et al., 2013). In contrast, the large molecules have low diffusivities and hence low surface diffusion, and also low pore volume diffusion, which is the rate limiting step (Leyva-Ramos et al., 2009). The pore volume diffusion rate is inversely proportional to the carbon tortuosity factor, and one of the GACs used (NRS) has a higher tortuosity factor than the PACs (SA2 and WP) (Xiao et al., 2015). Thus, the pore volume diffusion rate is lower in NRS than in SA2 and WP. This also means that the high MW compounds will take more time to diffuse into NRS than the PACs. Due to this slower diffusion of the large compounds, pore blockages in the GAC are less serious for the VFAs, so the small compounds can diffuse into the pore volumes and adsorb with a higher efficiency. One literature study indicated that pore blockage can result in a 10-99% reduction of the total capacity of ACs for chemicals (Ebied et al., 2001). The explanation suggested above is supported by the low adsorption of the high MW fraction (134 kDa) in GACs compared with other PACs in this case. Consequently,

the GAC will have a higher adsorption capacity toward VFAs than the PACs, and as result of the VFA adsorption in our case, the COD removal efficiency of these two activated carbons increased significantly (Fig. 7.2) as the COD_{VFA} contributes to a large fraction of the COD in this effluent; hence EFF-II resulted in a higher removal efficiency than EFF-III. The reason for this was due to a higher fraction of high MW compounds in EFF-II than in EFF-III which will cause greater pore blockage, and lead to less diffusion of VFAs into the pores.

Figure 7.5 VFA removal by ACs in AnMBR effluents: (a) EFF-II and (b) EFF-III. Each bar represents the average of three replicates, while the Error bars represent the standard deviation of the measurements.

7.3.4 Low-MW SMP removal

Different SMPs were identified with concentrations ranging from 0.01 to 49.31 $\mu\text{g/L}$. The highest concentration was p-cresol in EFF-III, followed by a few esters (methyl oleate, methyl stearate), and alkanes (tetradecane, hexadecane, octadecane) as shown in Table S7.1 (Appendix). The low-MW SMPs were classified as: alkanes, alkenes, cycloalkanes, acids, esters, nitrogen containing-compounds (N-compounds), phenols and some unidentified compounds. These group of compounds were also found in a recent study on AnMBR effluent (Kunacheva et al., 2017a, Kunacheva et al., 2017c). In the literature there is only one study which measured the removal of these SMPs compounds, and this demonstrated the effective removal of some N-compounds (m-Aminophenylacetylene), cycloalkanes (cyclohexane 1,2,4 trimethyl) and cholestan 3-one when using PAC to treat saline AnMBR effluent (Vyrides et al., 2010). In our study, after adsorption the different groups of SMP compounds showed different degrees of adsorption performance with different activated carbons, with phenols and N-compounds being approximately 100% removed in most cases (Fig. 7.6).

Figure 7.6 COD removal by ACs from AnMBR effluents: EFF-I (a), EFF-II (b), EFF-III (c), and EFF-IV (d). Each bar represents the average of removal of all SMPs compounds within a group, while the Error bars represent the standard deviation of the measurements.

Two phenol compounds, p-cresol and 3,5-Di-tert-butylphenol, showed 100% removal in most cases except with EFF-IV where it only showed 13 and 23% removal for 3,5-Di-tert-butylphenol removed by NRS and SA2, respectively (Table S7.1). The high adsorption capacity of p-cresol onto GAC was shown earlier in one study, with an optimum adsorption capacity of 12.02 mg/g (Das et al., 2012). The mechanism of adsorption of p-cresol onto activated carbon was mainly via chemisorption due to electron transfer. In addition, the phenolic compounds can be adsorbed via π - π interactions, hydrogen bonding, and a combination of these mechanisms (Das et al., 2012). The N-compound groups are highly adsorbed onto activated carbon, regardless of their differences in chemical structure, with removal efficiencies of mainly 100%. The adsorption of N-compounds was also shown to depend strongly on the type and amount of an oxygen functional group on the carbon surface (Almarri et al., 2009). Basic N-compounds such as quinoline are more adsorbable on acidic functional groups such as carboxyl and anhydride groups, whereas neutral N-compounds such as indole are more influenced by the basic carbonyl group (Almarri et al., 2009). Regardless of the different types of activated carbons used, and the difference in chemical structures of the N-compounds identified in this study, including indolics (Indole, 2-Indolinone), pyrazines (Pyrrolidino[1,2-a]piperazine-3,6-dione; 3-Benzylhexahydropyrrolo[1,2-a]pyrazine-1,4-dione; benzylpiperazine (Cyclo(leucylopropyl)), 3-Benzyl-piperazine-2,5-dione), and Benzonitriles (2,4,6-Trimethylbenzonitrile), the removal of N-compounds on activated carbon in this study was excellent.

In contrast, other groups such as alkanes, alkenes, cycloalkanes, esters had a lower removal efficiency (Fig. 7.6). Many compounds such as alkanes and esters, however, showed an increase in concentration as documented in Table S7.2 (Appendix). In addition, acids were 100% removed for the compound identified, although there was only one compound, 9-Hexadecenoic acid, identified in all four effluents. The appearance of many compounds, mostly alkanes, after adsorption at concentrations higher than in the initial effluent sample was an interesting observation. The blank test that analysed low-MW SMPs in ultrapure water and from the eluted activated carbon (dissolved in ultrapure water) were assessed; these showed the appearance of alkanes after stirring activated carbon into ultrapure water (results not show). Hence, one reason for this increase in alkanes could be the adsorption of alkanes onto the activated carbon when exposed to indoor air (Zhang et al., 2000). In addition, this literature study also

showed that GAC was not effective in removing alkanes at low ppb concentration ranges, which agrees with our study.

Besides the removal of low-MW SMPs, CIP was also shown to be effectively removed by activated carbons. Effluents had CIP concentrations of 2.7, 0.2 and 0.1 mg/L for EFF-II, EFF-III and EFF-IV, respectively. After adsorption, the treated effluents had CIP concentrations lower than the detection limit except for the cases of EFF-II with two activated carbons, FD and NRS, which only removed 75% and 98% CIP, respectively.

7.3.5 Contribution of the size fractions and specific compounds to COD removal

The reduction of COD was a combination of the different removal of different organic carbon fractions, and in this case it was characterized by the fractions including; 1522 kDa, 134 kDa, 72.8 kDa, 0.6 kDa, and < 0.1 kDa. The small compounds (< 580 Da) were analyzed specifically as VFAs and low-MW SMPs. However, these size fractions on SEC only showed the appearance and reduction of these fractions, but not their concentration, so no quantitative calculations on the contribution of each part to COD removal is shown here.

The removal of low-MW SMPs compounds did not significantly affect COD removal because of the low concentrations of these compounds in the effluents, and the total concentration of low-MW SMPs was estimated as 23, 15, 221, and 19 µg/L for EFF-I, EFF-II, EFF-III, and EFF-IV, respectively. The theoretical COD of these compounds can be calculated from their chemical formula (and reduction state), and the COD_{low-MW SMPs} could be equal to 1.9-3.5 times higher than the concentration of low-MW SMPs (Table 3.4). Even if the maximum removal efficiency was achieved for all of these SMPs, the COD removal of them would only contribute to less than 1.4%. Despite this consideration, many low-MW SMPs are toxicants, for example p-cresol is classified as a priority pollutant (US-EPA, 1999), and indoles, and p-cresol are potential carcinogens; hence the complete removal of these low-MW SMPs could be important not only for the environment, but also human health. Moreover, the removal of SMPs by activated carbon is also an effective treatment method for the reduction of disinfection by-products, and activated carbon removal of SMPs by 60% will lead to a concurrent reduction in disinfection by product formation potential (DBPFP) by more than 70% (Liu and Li, 2015).

The behavior of COD removal in this study can be explained by a significant reduction in VFAs combined with a reduction in the high MW fraction. With low COD effluents, and no VFAs present, for example EFF-IV, the reduction of COD was mainly due to the reduction in the 134 kDa fraction, and this fraction was the main reason for the difference in effectiveness of activated carbons also. Differences in particle size of the activated carbon affected the adsorption capacity more compared to the BET surface area, and GAC had a lower adsorption capacity than PAC, even when GAC had a similar or higher BET surface area than PACs.

When more high MW compounds and VFAs were present, COD reduction not only depended on the 134 kDa fraction, but also on other fractions, and especially on the reduction of VFAs. In EFF-II, when the high MW fraction was not very high, VFAs could be removed effectively, and this was the major reason for COD reduction. However, the reduction of VFAs was indirectly dependent on the type of activated carbon. Our suggested mechanism is that the activated carbons will firstly affect the reduction of the 134 kDa and 72.8 kDa fractions. However, activated carbons resulted in the complete removal of the 72.8 kDa fraction, but a different reduction in the 134 kDa fraction, which was the same trend in EFF-IV. After this the adsorption of these high MW-fractions competed with the adsorption of VFAs onto activated carbon, or it blocked the pores of the ACs and consequently lowered the adsorption capacity of the VFAs onto activated carbon. This meant that the activated carbons which had a high adsorption capacity for the high MW fraction will only achieve low VFA reduction, and hence the difference in COD removal between the ACs will be the result of differences in COD_{VFA} removal, and removal of the 134 kDa fraction.

In EFF-IV, since the presence of the high MW fraction was significant, with an increase in the 1,522 kDa fraction, and the appearance of a 0.6 kDa fraction, COD reduction would be the sum of combining the 3 size fractions (1,522 kDa; 134 kDa and 0.6 kDa) and VFAs. The removal of the 134 kDa fraction also depended on particle size, and similarly to previous cases, high MW adsorption would block the pores of ACs, leading to a significant reduction in VFA adsorption capacity. Since VFAs also contribute significantly to the effluent COD in this case, the total COD reduction was low and depended on the adsorption of high MW compounds (larger than 1 kDa) only.

7.4. Conclusion

Activated carbon adsorption is not very effective for the post-treatment of AnMBR effluents, and many fraction/compounds were shown not to readily adsorb. The mechanism of COD removal from AnMBR effluent by activated carbon is complex and depends strongly on certain properties of the activated carbon, primarily particle size, and the effluent composition, including the size distribution of the organic compounds (SMPs) and VFA concentrations. Total COD removal did not follow any specific trend for all the effluent types, but was different from case to case, as it is a combination of the removal of many different fractions and compounds. However, the difference in COD removal between different activated carbons was mostly due to a difference in the adsorption of compounds in the 134 kDa fraction, and in the < 1 kDa fraction, especially the VFA concentration. The removal of the low-MW SMPs compounds is not significant for COD reduction (due to their low overall concentration), but is significant for environmental protection and human health as they can greatly reduce the concentration of some toxic compounds, for example phenols. Phenolics and N-compounds in SMPs, and CIP, were greatly removed by adsorption, while other groups including esters, alkanes, alkenes, and cycloalkanes showed lower or no removal.

CHAPTER 8

Concluding Discussion, Conclusions and Future Work

Outline:

8.1 Introduction

8.2 Concluding discussion

8.3 Conclusions

8.4 Recommendations for future work

8.1 Introduction

The extent to which this work has met the initial objectives is analysed in this chapter. The important findings and the conclusion drawn from this study, are then presented. Finally, the areas of research that could be further explored, and the knowledge gaps that remain and need to be investigated in more detail are highlighted in Future Work.

8.2 Concluding discussion

The main objectives of this work were to investigate the influence of CIP at high but environmentally relevant concentrations on anaerobic batch reactor in general, and the AnMBR in particular, and the removal of CIP in an AnMBR. We also investigated the production of organic compounds when CIP was fed to the AnMBR, especially the production of SMPs and at what concentration CIP would have a significant effect. In addition, the shifts in microbial ecology, and the biodegradation pathways were also investigated. Lastly, the post-treatment of AnMBR effluent using activated carbon with the aim of removing low MW compounds was investigated. Based on these experimental findings, the following discussion of the study can be made.

8.2.1 Effect of CIP on the performance of the anaerobic reactor

It was generally thought that the antibiotic would have a significant effect on the performance of the bioreactor, at least at the level of mg/L. The literature showed the inhibition of many microorganisms at low concentrations of CIP, even at levels of a few µg/L. CIP posed a high ecological risk to various aquatic organisms, in which the most sensitive included *Microcystis aeruginosa* and *Lemna minor*, which had EC₅₀ toxicity values as low as 17 and 203 µg/L, respectively (Robinson et al., 2005). 31% of 362 strains of anaerobic bacteria were susceptible to CIP, with tentative breakpoints of 1 µg/mL (Sutter et al., 1985). That could be the reason why the operation of the bioreactor in the presence of antibiotics including CIP were generally avoided. However, this study showed that the anaerobic batch reactor and AnMBR could be operated with good performance even at CIP concentrations of < 0.5 mg/L and could function biologically even in the presence of high CIP concentrations up to 5 mg/L. Though, being an antibiotic, CIP still affected both the rate and levels of methane production, and these decreased as the CIP concentration increased, with a significant effect being observed even at 0.5 mg CIP/L ($p < 0.05$). The inhibitory concentration measured with the batch assays was IC₂₀ = 0.11, IC₅₀ = 4.8, IC₈₀ > 42.94 mg CIP/L. The operation of an AnMBR over the long-term with CIP in the feed showed no statistical difference in COD removal and biogas production, and no VFAs were observed when 0.5-1.5 mg/L of CIP was added for 52 days. However, only when 4.7 mg CIP/L was added to the feed did the COD removal reduce gradually, and the average COD removal under these conditions was $78 \pm 13\%$. At the same time, biogas production was lower and VFAs accumulated in the effluent. Hence, the long-term addition of CIP did not inhibit the performance of CIP at low CIP concentrations, and it was only at high concentrations of CIP (around 4.7 mg CIP/L) that inhibition was observed. However, when the addition of CIP was stopped, performance recovered, with higher COD removal efficiencies and methane production, while the VFA concentrations decreased. Thus, the study implied that the performance of an AnMBR under long term exposure to CIP was possible. This observation raises the potential of operating an AnMBR as well as other anaerobic reactors to treat wastewater contained CIP.

8.2.2 Effect of CIP on the characteristics of anaerobic effluent

The effect of operational conditions of a AnMBR on the performance and quality of effluent was demonstrated under different HRT, SRT, temperature, high organic loading

and the presence of suspended solids (Huang et al., 2011; Kunacheva et al., 2017c, Smith et al., 2015, Win et al., 2016, Watanabe et al., 2016). These data indicated that the COD concentration in the effluent would increase under unfavourable conditions such as under a pH shock of 5 or 11 (Kunacheva et al., 2017b), along with the increase in COD, an increase in VFA concentration was also recorded (Kunacheva et al., 2017b). In this study, the effect of an antibiotic on the change and characteristics of organic compounds inside the AnMBR and its effluent was evaluated. This showed that CIP affected COD removal, the size of organic compounds in the reactor and effluent, the accumulation of VFAs and low-MW SMPs, and a significant effect on these parameters was induced at 0.5 mg/L and higher. In the literature there have been very few studies on the size distribution of solutes in anaerobic reactors. The high MW fraction increased at both low or high pHs, an increase in SRT, and a decrease in alkalinity (Aquino and Stuckey, 2004; Kunacheva et al., 2017b; Kuo and Parkin, 1996). In a recent study, the size fraction of the organics showed that the highest concentration of carbohydrates (30 k–200 kDa) were found in the pH 5 shock, while the highest MW (1500 kDa–0.2 μ m) protein-like compounds were found under the pH 11 shock (Kunacheva et al., 2017b). This study showed the size distribution of the organic compounds inside the AnMBR ranged from $>>1522$ kDa to <100 Da. When a low CIP concentration was added to the AnMBR (0.5 mg/L), there was a small change in the MW distribution with the appearance of a peak at around 73 kDa. However, after feeding with a high CIP concentration (4.7 mg/L), these fractions increased significantly. The identify of compounds generated, and why/how they were produced, is unknown, but they are likely to be BAPs, which are produced because of cell decay and the turnover of cellular structures. The increase of high MW compounds as BAPs was suggested and demonstrated by determining the DNA/VSS and protein/SMP produced by lysis in an anaerobic chemostat under low alkalinity feed conditions (Aquino and Stuckey, 2004).

The effect of an antibiotic (CIP) in this study on low-MW SMPs is reported for the first time. The low-MW SMPs identified in this study belonged to various groups, including alkanes, alkenes, esters, alcohols, phenols, N-compounds, fatty acids and S-compounds. Except for fatty acids, these compounds have been shown to be present in the effluent of an AnMBR (Trzcinski and Stuckey, 2010). However, long chain acids such as 9-Hexadecenoic acid, 4-Methylhexanoic acid and Heptanoic acid were only produced with high concentrations of CIP (> 5 mg CIP/L in the AnMBR and 50 mg/L in the batch

reactor). Many of these SMPs have also been found in the headspace of pure strains of microorganisms (Citron et al., 2012), although the biosynthesis pathway of most of these compounds is not known. The number of N-compounds such as Indole, 1-isocyanato-2-methylbenzene, 1-(Benzthiazol-2-ylthio)-propan-2-one, 2,5-Piperazinedione, 3-(phenylmethyl) increased with an increase in CIP concentration. Noticeably, P-cresol was dominant at CIP concentrations >2.5 mg/L, and was 5-8 times higher than the second most abundant SMP (5-methyl-1H-Benzotriazole), and its concentration increased up to 49 µg/L. The dominance of p-cresol was noticeable in this study, but p-cresol has been identified in a previous study (Kunacheva et al., 2017b) but at low concentrations. Only with the influence of CIP was p-cresol increased significantly, dominating other SMPs; hence this raises a question about the metabolism producing p-cresol as well as other SMPs compounds under different operating conditions of the AnMBR.

Finally, some of SMPs are toxic or potentially toxic. For example, Indole and Cresol are known to be carcinogenic (Nowak and Libudzisz, 2006), and the disinfection by-products (DBPs) from SMPs could be more environmentally toxic than the SMPs themselves (Zhang et al., 2015). These DBPs are often genotoxic, mutagenic and/or carcinogenic, with obvious adverse effects on human health (Richardson et al., 2007), especially for N-compounds DBPs that could be more harmful than carbon-containing DBPs (Plewa et al., 2007). This raises the issue amongst researchers to look more closely at the presence of other SMPs in anaerobic effluents, as well as the potential effects of DBPs on humans and the ecosystem.

8.2.3 Effect of CIP on microorganisms

Anaerobic degradation is complex and involves a wide variety of different bacteria including hydrolytic, fermentative, acetogenic and methanogenic bacteria. Two pathways have been reported for methane production from acetate in methanogenesis to date (Petersen and Ahring, 1991, Schnürer et al., 1994). The first is direct methanogenesis by aceticlastic methanogenic archaea with *Methanosarcinales* members (Fournier and Gogarten, 2008, Vanwonterghem et al., 2016), with two known genera of methanogens *Methanosarcina* and *Methanosaeta* (Hattori, 2008). The second pathway is non-aceticlastic oxidation with the co-metabolic pathway of acetate-oxidizing bacteria and hydrogenotrophic methanogens, corresponding with *Clostridium* spp. and *Methanomicrobiales* or *Methanobacteriales*, respectively (Hattori

et al., 2000, Petersen and Ahring, 1991, Schnürer et al., 1997). Imbalance in the operation of anaerobic systems causes changes in the microorganism community structure, and this is manifest in either/or reduced methane production, increased VFA formation and increasing effluent CODs. It has been shown that two AnMBRs with different biomass origins and characteristics resulted in different performance and dominant bacterial species when they treated the same wastewater (Ng et al., 2015). Hence, it is impossible to predict the changes in the microorganism structure and how they respond as the microorganism community will not be the same when they come from different sources. In this study, the 16s rRNA sequencing showed CIP significantly altering the phylogenetic structure of the microbial community in the batch reactor when the communities were exposed to high CIP concentrations of 50 mg CIP/L. At the genus level, *Syntrophobacter*, *Aminomonas*, *Melioribacter*, *Geobacter*, and *Methanothrix* decreased significantly at >0.5 mg CIP/L. The reduction of *Syntrophobacter* could lead to the accumulation of propionate at high CIP concentrations, while the reduction of *Methanothrix* presumably resulted in the significant reduction in methane production. Moreover, the study indicated that the methane production pathway associated with the aceticlastic methanogenic archaea, *Methanothrix*, was inhibited when exposed to CIP. In contrast, *Methanobacterium*, a hydrogenotrophic bacteria, was significantly enriched at high CIP concentrations (>0.5 mg/L). Hence, our study indicated that in the two pathways producing methane from acetate, the hydrogenotrophic methane production route might be less disturbed at high CIP concentrations compared to those via aceticlastic methanogenesis.

8.2.4 Removal and fate of CIP in an anaerobic reactor

In the literature, the degradation of CIP in a biosystem has been reported, but not with any consistency; the degradation of CIP was reported in a few systems, but not in others. In these cases the removal efficiency was highly variable, ranging from 0-100%, and little detail on the fate and transport of CIP was reported (Michael et al., 2013). SRT is an important reactor parameter in the degradation of antibiotics as high SRT will enrich for slow-growing bacteria, providing a greater diversity in the microbiome, and thus enhancing the ability of the culture to degrade antibiotics. Membranes associated with the reactor will therefore achieve higher performance as its provide a high SRT by eliminating the washout of biomass. An aerobic MBR removed between 54-93% of CIP when treating hospital wastewater (Nguyen et al., 2017), however, the mechanism of

CIP removal was not examined in any depth. CIP was also shown to be biodegraded in several systems (Dorival-Garcia et al., 2013, Hamjinda et al., Li and Zhang, 2010, Liao et al., 2016, Liu et al., 2013a, Liu et al., 2013c), but not in other systems (Golet et al., 2003, Li and Zhang, 2010, Liu et al., 2013c). The AnMBR has shown good performance in treating pharmaceutical and chemical industries wastewater (Svojitka et al., 2017); however, no studies have investigated the treatment of antibiotics. Hence, before this study was carried out, understanding the removal of CIP in anaerobic reactors was poor, as well as their removal mechanisms. In this study, the anaerobic systems examined showed true biodegradability of CIP both in anaerobic batch reactors and the AnMBR. In batch reactors an average removal of CIP of around 85% (70-95%) was achieved after 12 days, and this efficiency increased over time. In a more detailed investigation into CIP removal, this study showed the amount of CIP adsorbed onto the biomass was strongly dependent on the concentration of CIP added, and varied between 2.6 and 462 mg CIP /kg dry weight. This value corresponded to 8-47% of the CIP removal, with the remaining fraction removed through biodegradation in the anaerobic batch reactor, although no degradation pathway could be postulated due to the lack of intermediate compounds information. In the AnMBR, 50-76% removal was achieved when 0.5-1.5 mg/L of CIP was added to the feed. At 4.7 mg CIP/L, the CIP removal gradually reduced to < 20%, and flattened out after 20 days. A mass balance showed that biotransformation was the dominant mechanism for CIP removal when low CIP concentrations (0.5-1.5 mg/L) were added to the feed. At higher concentrations AnMBR performance was significantly affected, with biological degradation/transformation reduced significantly, and most of the CIP was discharged with the reactor effluent. The removal by adsorption was low, generally < 5%, except for a short period when it contributed up to 26% of the total removal. In the literature, twenty-four CIP transformation products were detected in a two-stage aerobic/anoxic MBR (Hamjinda et al., 2017), and a few of these were also found in this study in the effluent of the AnMBR when a high concentration of CIP was fed. An intermediate with a MW of 305 was produced due to defluorination, and another with a MW of 329 was a product of the oxidation of the amine moiety. In addition, 3,4-Dimethyl-2(1H)-quinolinone could have potentially been an intermediate, but the degradation pathway is still unknown. Thus, this study confirmed the removal of CIP in either an anaerobic batch reactor or AnMBR with the main removal pathway being biodegradation. In addition, a few other degradation routes were found such as the defluorination and the oxidation of the amine moiety. This contributed to a greater

depth in understanding in the biodegradation pathway of CIP removal in anaerobic reactors, and a potential treatment process to treat wastewater containing CIP.

8.2.5 Post treatment of AnMBR effluent by activated carbons

The use of activated carbon for water treatment has been used for a long time due to its high adsorption capacity resulting from its highly porous structure with a large surface area (500-1,500 m²/g), and numerous internal pores and functional surface groups. Due to this, activated carbon can adsorb various solutes that are known to be present in water, including organics such as phenol and phenolic compounds (Dąbrowski et al., 2005), volatile organics (Chiang et al., 2002) and pharmaceuticals (Rakić et al., 2015). However, studies on the adsorption by activated carbon were generally focused on overall removal efficiency, and on the removal of a few known organic compounds that were spiked into the water. Up to now, characterisation and removal of specific organic compounds or SMPs was limited and not fully understood due to the inadequacy of existing analytical methods, although adsorption by PAC and GAC was found to be one of the most efficient treatment methods for removing traces of organic solutes for the post-treatment of AnMBR effluents (Trzcinski et al., 2011; Vyrides et al., 2010). In this study, the post treatment of AnMBR effluent by activated carbon achieved low to moderate COD removal (22-82%) at a dose of 1 g activated carbon/L of AnMBR effluent. Activated carbon adsorption does not remove all organic compounds, and Gur-Reznik et al. (2008) showed that about 24 % of the dissolved organic matter was non-adsorbable, being mainly hydrophilic and transphilic compounds, although the exact composition of these compounds is unknown due to analytical challenges. This study showed no direct correlation between COD removal and BET surface area, pore volume and pore size, but seemed to depend on the tortuosity factor of the activated carbon.

The size (MW) of the organic compounds was shown to normally be a factor that affects the adsorption onto activated carbon; the high MW organics in anaerobic effluent were shown to be more adsorbable than low-MW compounds (Barker et al., 1999; Trzcinski et al., 2011; Vyrides et al., 2010). The < 1 kDa fraction was only 70% removed by PAC, while higher fractions showed higher removal efficiencies of 90-100% (Vyrides et al., 2010). However, the work in this thesis also showed that the chemistry of these organics was also an important factor determining the removal efficiency of the adsorption process. The organic compounds in the AnMBR effluents had size distributions from < 0.1 kDa to 1,522 kDa; after adsorption by activated carbon, the 134 kDa and < 0.1 kDa

fractions were difficult to remove, whereas the 1522 kDa, 73 kDa and 0.6 kDa fractions were significantly removed. Characterization of these fractions showed that the 134 kDa fraction could be a mixture of many polymeric compounds, including aromatics, proteins and carbohydrates, while the < 0.1 kDa fraction was most likely carbohydrates. However, of the MW compounds of 1522 kDa, 73 kDa and 0.6 kDa which decreased significantly, most were primarily aromatic compounds. Hence, the size of the organic compounds was not the only factor to determine their removal by adsorption, but also the functional groups of the organic compounds.

When studying in depth the removal of specific organic compounds by AC, this work showed VFA removal affected COD reduction significantly as VFAs contributed to a large percentage of the effluent COD. The AnMBR effluent characteristics showed a significant influence due to the removal of acetate and propionate; acetate and propionate dominated the VFAs and could be very efficiently removed (up to 100%), however, this depended on AnMBR effluent characteristics and AC type. Surprisingly, two GACs, FD and HYD, showed noticeably better performance than PACs for VFA adsorption. The study also demonstrated that the presence of a high MW fraction in the effluent, and the tortuosity factor of the activated carbon, corresponding with the diffusion rate, could be the main reasons for this difference.

Different groups of SMP compounds showed different degrees of adsorption with different activated carbons. Phenols and N-compounds were 100% removed in most cases, while alkanes, alkenes, cycloalkanes, and esters had a lower removal rate, however, some compounds such as alkanes and esters, showed an increase in concentration. The removal of low-MW SMPs compounds did not significantly affect COD removal; however, they are beneficial in terms of removing toxicants, for example p-cresol is classified as a priority pollutant (US-EPA, 1999), indoles and p-cresol are potential carcinogens, and the remaining antibiotic CIP. CIP was also shown to be effectively removed (> 99%) from the AD effluent using most of the activated carbons tested for effluents taken during different operational conditions, except for two GACs (FD and NRS) which showed very little removal when treating high concentration CIP effluent (2.7 mg/L). Hence, in addition to the performance of AC in removing organic compounds, the study showed in more depth the removal of some size fractions of organic compounds and specific compounds including VFAs and low-MW SMPs. The study also lays down a basis for predicting which AC is best for treating different types

of wastewater when we know their characteristics, including size fractions of the organic compounds, and VFA concentrations.

8.3 Conclusions

Based on the results obtained from this study, the following conclusions can be drawn:

- CIP affected anaerobic batch reactors from concentration of >0.5 mg/L which was manifest in various parameter including COD removal, VFA and SMPs accumulation, biogas production, and a shift in microbial community structure.
- The long-term effect of 0.5-1.5 mg/L CIP on AnMBR performance was not significant, however, at 4.7 mg/L there was a significant effect on all the parameters tested.
- More N-compounds were produced when anaerobic batch reactor and the AnMBR were exposed to CIP. P-cresol appeared and dominated other low-MW SMPs compounds at high CIP concentrations.
- *Syntrophobacter* and *Methanothrix*, which carry out acetogenic and acetoclastic methanogenesis were shown to be affected by CIP at concentrations between 0.5-50 mg/L.
- 50-76% CIP removal occurred when <1.5 mg/L of CIP was added to an AnMBR feed. At a higher CIP concentration, 4.7 mg/L, the removal efficiency decreased gradually to $< 20\%$ removal.
- Biological transformation/degradation was the main route of removing CIP in an AnMBR when the performance of AnMBR was not disturbed by its exposure to high concentrations of CIP. High CIP concentrations (> 1.5 mg/L) and the lower performance of the AnMBR resulted in much lower CIP biodegradation, leading to a lowering of total removal efficiency.
- VFAs could be removed by adsorption onto ACs, and their removal contributed significantly to COD removal. The size fraction (MW) is not necessarily the major characteristic of organics in predicting the adsorption of organic compounds, but their chemical properties do help us predict their adsorbability.

- N-compounds, and phenolic compounds belonging to the category of SMPs, and CIP were almost completely removed by activated carbon, reaching 100% removal. Alkanes, alkenes, and esters were more difficult to remove.

8.4 Recommendations for future work

Based on the findings of this research, and issues raised throughout this project, the following recommendations for future study are suggested:

- Study the synergistic effects of CIP and other antibiotics on anaerobic digestion in general, and the AnMBR in particular. The effect of CIP on anaerobic digestion and AnMBR have been studied thoroughly here, however, it is known that different antibiotics have a common mechanism of action that will have a greater impact in mixture and cause additive or synergistic effects resulting in a greater impact than the individual compounds. Hence future research is required to determine whether synergistic effects with antibiotics will enhance the adverse effects to anaerobic digestion at low concentrations of each antibiotic. The work should examine how low the concentration of each compound will be to affect methanogenic. In addition, the organic compounds (SMPs) released should be characterized, especially low-MW SMPs, and the shift in microbial ecology determined to see whether the effect of combined antibiotics differ to the effect caused by CIP alone.
- Further characterize the organic compounds (SMPs) in the effluent. More techniques such as LC-MS/MS, and Matrix Assisted Laser Desorption Ionization-Time of Flight-Mass Spectrometry (MALDI-ToF-MS) should be used to identify more of the compounds present in solution. LC-MS/MS can detect polar and thermo-unstable compounds, while MALDI-ToF-MS could be useful for detecting high-MW SMP compounds.
- Investigate the feasibility of using biological activated carbon for the post-treatment of AnMBR effluent. Mixing biomass with GAC before adding it into the GAC columns should be assessed to determine the potential of biological activated carbon (BAC) in the post-treatment of AnMBR effluent. Developing specific microorganisms on activated carbon before starting the adsorption

column is hypothesised to extend the life of the activated carbon due to biological regeneration of the activated carbon. Different inoculum such as anaerobic or aerobic biomass should be used and compared.

- The removal and recovery of phosphorus from AnMBR effluents. During AnMBR treatment, the removal of Phosphorus is not significant, and is the reason why post-treatment of Phosphorus may be needed as well for the recovery of this nutrient. Adsorption by a hybrid anion exchanger (HAIX) or by algae would be the potential methods of choice. A hybrid anion exchanger containing hydrous ferric oxide will increase the sorption capacity of phosphate, and can be regeneration and reused to recover phosphorus, and since the phosphorus is concentrated it can be used as a nutrient. Algae can also take up nutrients to produce biomass, and this is a valuable oil (bio diesel) source that has been shown to have a higher potential for renewable biodiesel production than existing oilseed crops (Schenk et al., 2008).

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APPENDIX

Table S4.1 List of SMPs compounds found in anaerobic digestion under CIP exposure.

No.	Similar index	Retention Index	Name	Mass	Formula	Retention time
1	93	1015	Decane	142	C ₁₀ H ₂₂	11.45
2	95	1115	Undecane	156	C ₁₁ H ₂₄	11.61
3	98	1014	p-Cresol	108	C ₇ H ₈ O	14.22
4	95	1115	Undecane	156	C ₁₁ H ₂₄	14.64
5	92	1109	Hexanoic acid, 2-ethyl-	144	C ₈ H ₁₆ O ₂	15.40
6	98	1092	Benzenamine, N-ethyl-	121	C ₈ H ₁₁ N	15.55
7	97	1173	Octanoic acid	144	C ₈ H ₁₆ O ₂	16.82
8	98	1214	Dodecane	170	C ₁₂ H ₂₆	17.20
9	98	1208	Benzothiazole	135	C ₇ H ₅ NS	17.94
10	94	1249	Benzeneacetic acid	136	C ₈ H ₈ O ₂	18.50
11	94	1272	Nonanoic acid	158	C ₉ H ₁₈ O ₂	18.87
12	97	1174	Indole	117	C ₈ H ₇ N	19.52

13	91	-	Benzene, 1-isocyanato-2-methyl-	133	C8H7NO	19.68
14	93	-	1H-Benzotriazole, 5-methyl-	133	C7H7N3	19.74
15	93	1349	Hydrocinnamic acid	150	C9H10O2	20.34
16	92	1448	Tetradecane, 5-methyl-	212	C15H32	20.62
017	92	1372	n-Decanoic acid	172	C10H20O2	20.92
18	92	2045	Eicosane, 10-methyl-	296	C21H44	20.97
19	94	1818	9-Octadecene, (E)-	252	C18H36	21.41
20	96	1413	Tetradecane	198	C14H30	21.56
21	95	1402	Dodecanal	184	C12H24O	21.79
22	95	-	1H-Benzotriazole, 5-methyl-	133	C7H7N3	23.14
23	91	1555	Phenol, 3,5-bis(1,1-dimethylethyl)-		C14H22O	23.72
24	94	1555	Phenol, 2,4-bis(1,1-dimethylethyl)-	206	C14H22O	23.81
25	91	1945	Nonadecane, 9-methyl-	282	C20H42	24.44
26	90	2045	Eicosane, 10-methyl-	296	C21H44	24.44
27	89	1448	Tetradecane, 5-methyl-	212	C15H32	24.45

28	96	1570	Dodecanoic acid	200	C ₁₂ H ₂₄ O ₂	24.65
29	92	1548	Pentadecane, 3-methyl-	226	C ₁₆ H ₃₄	24.78
30	94	1612	Hexadecane	226	C ₁₆ H ₃₄	25.29
31	90	1320	Dodecane, 2,6,11-trimethyl-	212	C ₁₅ H ₃₂	25.32
32	92	1548	Benzothiazole, 2-(methylthio)-	181	C ₈ H ₇ NS ₂	25.61
33	94	1670	Tridecanoic acid	214	C ₁₃ H ₂₆ O ₂	26.35
34	94	1351	2(3H)-Benzothiazolone	151	C ₇ H ₅ NOS	26.70
35	92	2804	Octacosane	394	C ₂₈ H ₅₈	27.84
36	92	2080	Eicosane, 2,4-dimethyl-	310	C ₂₂ H ₄₆	27.84
37	92	2046	2-Nonadecanone	282	C ₁₉ H ₃₈ O	28.07
38	97	1404	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	154	C ₇ H ₁₀ N ₂ O ₂	28.15
39	93	1779	Pentadecanoic acid, methyl ester	256	C ₁₆ H ₃₂ O ₂	28.46
40	94	1612	Hexadecane	226	C ₁₆ H ₃₄	28.61
41	91	1814	Pentadecanoic acid, 14-methyl-, methyl ester		C ₁₇ H ₃₄ O ₂	30.02
42	95	1886	Methyl Z-9-hexadecenoate	268	C ₁₇ H ₃₂ O ₂	30.28

43	92	2085	9-Octadecenoic acid (Z)-, methyl ester	296	C19H36O2	30.28
44	95	1878	Hexadecanoic acid, methyl ester	270	C17H34O2	30.57
45	93	1699	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	210	C11H18N2O2	30.81
46	90	1883	1-(Benzthiazol-2-ylthio)-propan-2-one	223	C10H9NOS2	30.92
47	95	1878	Hexadecanoic acid, methyl ester	270	C17H34O2	31.02
48	92	2225	Stearyl iodide	380	C18H37I	31.22
49	93	1501	2-Mercaptobenzothiazole	167	C7H5NS2	31.36
50	92	2009	Eicosane	282	C20H42	31.61
51	93	2085	11-Octadecenoic acid, methyl ester	296	C19H36O2	33.17
52	93	2077	Methyl stearate	298	C19H38O2	33.42
53	90	2080	Eicosane, 2,4-dimethyl-		C22H46	33.71
54	92	1780	2,5-Piperazinedione, 3-(phenylmethyl)-	204	C11H12N2O2	34.23
55	93	2208	Docosane	310	C22H46	34.36
56	90	2138	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	244	C14H16N2O2	36.43

57	90	2407	Tetracosane	338	C24H50	36.88
58	92	2138	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	244	C14H16N2O2	36.98
59	90	2506	Pentacosane	352	C25H52	38.06
60	96	2606	Hexacosane	366	C26H54	39.20
61	90	2705	Heptacosane	380	C27H56	40.30
62	92	2804	Octacosane	394	C28H58	41.35
63	93	2914	Squalene	410	C30H50	41.77
64	94	2840	Nonacosane	408	C29H60	42.37
65	94	2904	Triacontan	423	C30H62	43.36
66	94	3202	Dotriacontane	450	C32H66	44.311
67	91	2586	Cholestan-3-ol, (3.beta.,5.beta.)-	388	C27H48O	44.64
68	91	2641	2-methylhexacosane	380	C27H56	45.00

Table S7.1 The removal of some low-MW SMPs compounds by activated carbons.

RT	Compound name	Chemical structure	Effluent type	Initial Concentration (µg/L)	Removal efficiency (%)					
					NRS	FD	SAS	SA2	WP	HYD
Acids										
31.5	9-Hexadecenoic acid	C ₁₆ H ₃₀ O ₂	EFF-III	0.62	100	100	100	100	100	100
Alkanes										
15.27	Undecane	C ₁₁ H ₂₄	EFF-II	0.38	28	18	16	20	20	52
15.27	Undecane	C ₁₁ H ₂₄	EFF-III	1.23	0	0	0	0	0	0
15.28	Undecane	C ₁₁ H ₂₄	EFF-I	2.24	27	15	59	58	52	55
15.29	Undecane	C ₁₁ H ₂₄	EFF-IV	2.98	0	11	0	0	2	55
17.85	Dodecane	C ₁₂ H ₂₆	EFF-III	10.78	93	2	36	83	92	87
17.85	Dodecane	C ₁₂ H ₂₆	EFF-II	0.30	0	0	0	0	0	0
21.61	Heptadecane	C ₁₇ H ₃₆	EFF-III	3.72	100	9	38	94	100	94
21.62	Heptadecane	C ₁₇ H ₃₆	EFF-II	0.03	0	0	0	0	0	0
22.21	Tetradecane	C ₁₄ H ₃₀	EFF-III	25.28	98	7	34	89	97	91

22.21	Tetradecane	C ₁₄ H ₃₀	EFF-II	0.20	0	0	0	0	0	0
22.22	Tetradecane	C ₁₄ H ₃₀	EFF-IV	0.76	0	0	0	0	0	100
24.98										
9	2-Methyloctadecane	C ₁₉ H ₄₀	EFF-III	0.43	100	50	49	100	100	100
25.44	3-Methylpentadecane	C ₁₆ H ₃₄	EFF-III	6.49	100	4	26	87	100	88
25.95	Hexadecane	C ₁₆ H ₃₄	EFF-III	18.66	96	1	27	78	94	84
25.96	Hexadecane	C ₁₆ H ₃₄	EFF-II	0.29	0	0	0	0	0	0
28.84	10-Methylnonadecane	C ₂₀ H ₄₂	EFF-II	0.17	0	0	0	0	0	0
28.84	3-Methylheptadecane	C ₁₈ H ₃₈	EFF-III	4.10	96	3	32	67	92	75
29.29	Octadecane	C ₁₈ H ₃₈	EFF-III	11.87	90	15	15	61	84	73
29.3	7-Methylheptadecane	C ₁₈ H ₃₈	EFF-II	0.53	0	0	0	0	0	0
29.30										
2	Octadecane	C ₁₈ H ₃₈	EFF-IV	0.49	0	0	0	0	0	100
29.78	2-Methyloctadecane	C ₁₉ H ₄₀	EFF-II	0.24	100	100	100	100	100	100
31.9	10-Methylnonadecane	C ₂₀ H ₄₂	EFF-III	2.11	82	19	18	56	70	63
32.3	Eicosane	C ₂₀ H ₄₂	EFF-III	5.48	84	24	18	48	76	68
32.31	Eicosane	C ₂₀ H ₄₂	EFF-II	0.22	0	0	0	0	0	0
32.32	10-Methylnonadecane	C ₂₀ H ₄₂	EFF-IV	0.56	0	0	0	0	0	100
34.68	2-methyltetracosane	C ₂₅ H ₅₂	EFF-III	0.57	100	32	17	68	73	53
35.05	Docosane	C ₂₂ H ₄₆	EFF-III	1.58	83	37	71	49	80	71

35.05											
9	Tritriacontane	C ₃₃ H ₆₈	EFF-IV	0.21	0	0	0	0	0	100	
37.56											
6	2-methyltetracosane	C ₂₅ H ₅₂	EFF-III	0.49	100	100	100	100	100	100	
-	-	-	EFF-III	0.90	100	100	100	100	100	100	
-	-	-	EFF-III	0.38	100	100	100	100	100	100	
42.04											
1	2-methylhexacosane	C ₂₇ H ₅₆	EFF-III	0.38	100	100	100	100	100	100	
-	-	-	EFF-III	0.25	100	100	100	100	100	100	
Alkenes											
22.05	(E)-3-Tetradecene	C ₁₄ H ₂₈	EFF-III	3.09	100	17	44	100	100	96	
25.83	(Z)-3-Hexadecene	C ₁₆ H ₃₂	EFF-III	4.10	100	7	35	88	96	89	
29.19	(E)-9-Octadecene	C ₁₈ H ₃₆	EFF-III	1.49	100	0	40	75	100	72	
25.72	5-Ethyl-1-nonene	C ₁₁ H ₂₂	EFF-III	0.31	100	0	100	100	100	100	
25.83	1-Tridecene	C ₁₃ H ₂₆	EFF-II	0.04	0	0	0	0	0	0	
29.19	1,37-Octatriacontadiene	C ₃₈ H ₇₄	EFF-II	0.03	0	0	0	0	0	0	
32.21											
8	(9E)-9-Icosene	C ₂₀ H ₄₀	EFF-III	0.32	100	100	100	100	100	100	
38.60											
2	1-Docosene	C ₂₂ H ₄₄	EFF-III	0.41	100	100	100	100	100	100	

40.91											
6	-	-	EFF-III	0.18	100	100	100	100	100	100	100
Cyclo-alkanes											
23.22	Nonylcyclohexane	C ₁₅ H ₃₀	EFF-III	3.86	100	9	24	83	100	84	
23.22	Nonylcyclohexane	C ₁₅ H ₃₀	EFF-II	0.03	0	0	0	0	0	0	
27.02											
4	Decylcyclohexane	C ₁₆ H ₃₂	EFF-III	0.47	100	18	0	100	100	100	
Esters											
	1-Ethyltridecyl										
25.45	phenylacetate	C ₂₃ H ₃₈ O ₂	EFF-II	0.14	100	100	100	100	100	100	
	1,3-Pentanediol,	2,2,4-									
26.02	trimethyl-,	dEFF- C ₁₆ H ₃₀ O ₄									
1	Isobutyrate		EFF-I	0.46	14	0	0	0	0	0	
	1-Isobutyl 4-isopropyl 3-										
26.03	isopropyl-2,2-	C ₁₆ H ₃₀ O ₄	EFF-III	1.68	47	24	56	64	73	48	
	dimethylsuccinate										
	2,2,4-Trimethylpentanediol-										
26.04	1,3-dEFF-Isobutyrate	C ₁₆ H ₃₀ O ₄	EFF-II	0.33	11	59	67	52	45	11	
26.05	1-[2-(Isobutyryloxy)-1-										
	methylethyl]-2,2-	C ₁₆ H ₃₀ O ₄	EFF-IV	1.94	60	66	49	72	76	100	

	dimethylpropyl	2-									
	methylpropanoate										
28.10	Methyl	15-									
1	methylhexadecanoate		C ₁₈ H ₃₆ O ₂	EFF-I	0.27	100	100	100	100	100	100
29.15	Methyl	17-									
2	methyloctadecanoate		C ₂₀ H ₄₀ O ₂	EFF-IV	0.27	100	100	100	100	100	100
29.37	Methyl	(7E)-7-									
7	hexadecenoate		C ₁₇ H ₃₂ O ₂	EFF-III	0.34	100	100	20	0	0	0
29.71	Methyl decanoate		C ₁₁ H ₂₂ O ₂	EFF-I	0.56	55	100	100	100	100	100
29.72	Methyl	13-									
1	cyclopentyltridecanoate		C ₁₉ H ₃₆ O ₂	EFF-III	0.49	100	70	0	0	0	0
30.51											
5	DEFF-Isobutyl phthalate		C ₁₆ H ₂₂ O ₄	EFF-I	0.14	0	0	0	9	0	0
30.52	Butyl 2-ethylhexyl phthalate		C ₂₀ H ₃₀ O ₄	EFF-III	0.81	0	37	48	59	22	12
30.53	DEFF-Isobutyl phthalate		C ₁₆ H ₂₂ O ₄	EFF-II	0.39	50	80	87	71	67	78
30.54	Isobutyl phthalate		C ₁₆ H ₂₂ O ₄	EFF-IV	1.46	69	48	43	81	58	74
	Methyl	15-									
30.69	methylhexadecanoate		C ₁₈ H ₃₆ O ₂	EFF-III	0.35	100	100	0	100	0	100
30.96	Methyl oleate		C ₁₉ H ₃₆ O ₂	EFF-I	11.52	17	41	99	100	97	96
30.97	Methyl oleate		C ₁₉ H ₃₆ O ₂	EFF-III	29.74	85	76	0	0	20	54
30.97	Methyl oleate		C ₁₉ H ₃₆ O ₂	EFF-II	0.58	0	49	94	93	94	89

30.99	Methyl palmitoleate	C ₁₇ H ₃₂ O ₂	EFF-IV	2.76	77	42	100	100	88	100
31.24	Methyl hexadecanoate	C ₁₇ H ₃₄ O ₂	EFF-I	5.32	25	53	100	100	98	94
31.26	Methyl stearate	C ₁₉ H ₃₈ O ₂	EFF-III	9.25	77	54	0	19	37	64
	Methyl	17- C ₂₀ H ₄₀ O ₂								
31.26	methyloctadecanoate		EFF-II	0.47	0	50	100	92	92	52
31.27	Methyl hexadecanoate	C ₁₇ H ₃₄ O ₂	EFF-IV	1.79	70	48	100	100	86	100
31.95	Butyl phthalate	C ₁₆ H ₂₂ O ₄	EFF-II	0.34	48	88	89	74	81	96
31.95	Butyl octyl phthalate	C ₂₀ H ₃₀ O ₄	EFF-III	0.51	0	8	100	48	100	14
31.96	Dibutyl phthalate	C ₁₆ H ₂₂ O ₄	EFF-IV	1.01	87	46	62	87	70	87
32.41	Methyl oleate	C ₁₉ H ₃₆ O ₂	EFF-III	1.03	8	0	0	0	0	0
	Methyl 4-(1,5-dimethyl-3-oxohexyl)-1-cyclohexene-1-carboxylate	C ₁₆ H ₂₆ O ₃								
32.81	carboxylate		EFF-II	0.18	15	75	100	100	100	84
	Methyl 4-(1,5-dimethyl-3-oxohexyl)-1-cyclohexene-1-carboxylate	C ₁₆ H ₂₆ O ₃								
32.82	carboxylate		EFF-IV	1.09	100	100	100	100	100	100
33.85	Methyl oleate	C ₁₉ H ₃₆ O ₂	EFF-I	2.07	44	53	100	100	100	100
33.87	Methyl 11-octadecenoate	C ₁₉ H ₃₆ O ₂	EFF-III	13.62	84	60	0	0	0	0
33.87	Methyl oleate	C ₁₉ H ₃₆ O ₂	EFF-II	0.13	0	70	100	100	100	100
33.88										
2	Methyl oleate	C ₁₉ H ₃₆ O ₂	EFF-IV	0.41	100	100	100	100	100	100

34.99	Benzyl carbazate	C ₈ H ₁₀ N ₂ O ₂	EFF-III	1.09	100	100	57	100	100	100
N-compounds										
	2-(Cyanomethoxy)-1-(4-hydroxybenzyl)-2-	C ₁₂ H ₁₂ N ₂ O ₅								
34.11	oxoethylcarbamic acid		EFF-II	0.16	100	100	100	100	100	100
24.03										
8	2,4,6-Trimethylbenzonitrile	C ₁₀ H ₁₁ N	EFF-III	0.39	100	100	100	100	100	100
23.79										
3	2-Indolinone	C ₈ H ₇ NO	EFF-IV	0.13	100	100	100	100	100	100
23.80	2-Indolinone	C ₈ H ₇ NO	EFF-II	0.36	100	100	100	100	100	100
	3-Benzoylpiperazine-2,5-									
34.99	dione	C ₁₁ H ₁₂ N ₂ O ₂	EFF-II	0.06	100	100	100	100	100	100
	3-Benzylhexahydro									
	pyrrolo[1,2-a]pyrazine-1,4-	C ₁₄ H ₁₆ N ₂ O ₂								
37.20	dione		EFF-II	0.28	100	100	100	100	100	100
37.74	-	-	EFF-II	0.09	100	100	100	100	100	100
	3-Methyl-1,4-									
	diazabicyclo[4.3.0]	C ₁₀ H ₁₄ N ₂ O ₃								
28.05	nonan-2,5-dione, N-acetyl-		EFF-II	0.06	100	100	100	100	100	100
28.44	-	-	EFF-II	0.04	100	100	100	100	100	100

	5,10-Diethoxy-2,3,7,8									
31.78	-tetrahydro-1H,6H-dipyrrolo[1,2-a:1,2-d]pyrazine	C ₁₄ H ₂₂ N ₂ O ₂	EFF-II	0.71	100	88	100	100	100	100
30.18	-	-	EFF-II	0.71	100	93	100	100	100	100
31.54	Cyclo(leucylopropyl)	C ₁₁ H ₁₈ N ₂ O ₂	EFF-II	0.52	100	94	100	100	100	100
31.68	-	-	EFF-II	0.58	100	89	100	100	100	100
	Hexahydropyrrolo									
28.77	[1,2-a]pyrazine-1,4-dione	C ₇ H ₁₀ N ₂ O ₂	EFF-II	0.45	100	100	100	100	100	100
20.19	Indole	C ₈ H ₇ N	EFF-II	0.09	100	100	100	100	100	100
29.31	N-Butyl benzenesulfonamide	C ₁₀ H ₁₅ NO ₂ S	EFF-IV	0.75	100	100	100	100	100	100
34.1	Tyrosine, N-carboxy-, cyanomethyl ester	C ₁₂ H ₁₂ N ₂ O ₅	EFF-III	0.77	100	100	100	100	100	100
	Phenols									
14.73	p-Cresol	C ₇ H ₈ O	EFF-III	49.31	100	100	100	100	100	100
14.77	p-Cresol	C ₇ H ₈ O	EFF-II	4.43	100	100	100	100	100	100
24.48	3,5-Di-tert-butyl phenol	C ₁₄ H ₂₂ O	EFF-IV	0.53	13	100	100	23	100	100
24.45	2,6-Di-tert-butyl									
4	phenol	C ₁₄ H ₂₂ O	EFF-I	0.30	100	100	100	100	100	100

Unknown										
17.86										
6	-	-	EFF-IV	1.06	0	0	0	0	0	100
24.46										
5	-	-	EFF-III	0.51	100	26	27	32	100	100
25.60										
8	-	-	EFF-III	0.35	100	100	100	100	100	100
34.66										
5	-	-	EFF-III	0.98	100	100	100	100	100	100
35.05	-	-	EFF-II	0.06	100	100	100	0	0	100
36.04	-	-	EFF-II	0.05	100	100	100	100	100	100

Table S7.2 List of compounds that release from activated carbon.

RT	Compound name	Chemical structure	Group	NRS	FD	SAS	SA2	WP	HYD
17.14	3-Methylundecane	C ₁₂ H ₂₆	Alkanes	-	-	-	-	-	0.15
17.66	(E)-3-Tetradecene	C ₁₄ H ₂₈	Alkanes	-	-	-	-	-	0.15
17.84	Dodecane	C ₁₂ H ₂₆	Alkanes	2.58	3.72	0.81	0.61	0.77	0.66
18.95	Dimethyl hexanedioate	C ₈ H ₁₄ O ₄	Esters	0.46	-	-	-	-	-
21.26	Hexadecane	C ₁₆ H ₃₄	Alkanes	-	0.25	-	-	-	-
21.61	-	-	Unknown	-	0.36	0.08	-	-	-
21.63	Heptadecane	C ₁₇ H ₃₆	Alkanes	3.42	-	1.44	3.52	0.14	-
21.63	Pentadecane	C ₁₅ H ₃₂	Alkanes	0.07	-	-	-	-	-
22.05	(Z)-3-Tetradecene	C ₁₄ H ₂₈	Alkenes	-	0.38	0.09	-	-	-
22.06	1-Heptacosanol	C ₂₇ H ₅₆ O	Alcohols	-	-	-	-	0.19	0.97
22.06	(E)-3-Tetradecene	C ₁₄ H ₂₈	Alkenes	0.23	-	-	0.51	-	-
22.07	(Z)-3-Hexadecene	C ₁₆ H ₃₂	Alkenes	2.76	-	0.29	2.27	-	-
22.20	Tetradecane	C ₁₄ H ₃₀	Alkanes	2.85	3.43	1.86	1.16	1.22	0.68
	2,4,7,9-Tetramethyl-5-decyn-								
22.53	4,7-diol	C ₁₄ H ₂₆ O ₂	Alcohols	-	0.34	-	-	-	-
23.22	Isopropylcyclohexane	C ₉ H ₁₈	Cycloalkanes	-	-	0.12	0.14	0.09	-
23.24	Nonylcyclohexane	C ₁₅ H ₃₀	Cycloalkanes	2.87	-	1.81	2.97	0.47	-
24.46	3,5-Di-tert-butylphenol	C ₁₄ H ₂₂ O	Phenols	-	0.16	0.06	-	-	-

25.10	Hexadecane	C ₁₆ H ₃₄	Alkanes	-	0.30	-	-	-	-
25.11	Hexatriacontane	C ₃₆ H ₇₄	Alkanes	0.27	-	-	-	-	-
25.44	3-Methylpentadecane	C ₁₆ H ₃₄	Alkanes	0.51	0.17	0.26	1.00	0.51	1.56
25.44	3-Methylhexadecane	C ₁₇ H ₃₆	Alkanes	0.38	0.23	0.20	-	0.28	-
25.45	3-Methylpentadecane	C ₁₆ H ₃₄	Alkanes	4.70	-	2.30	5.09	1.05	-
Heptadecafluorononanoic acid,									
25.82	undecyl ester	C ₂₀ H ₂₃ F ₁₇ O ₂	Esters	-	0.28	-	-	-	-
25.84	(E)-9-Octadecene	C ₁₈ H ₃₆	Alkanes	1.61	-	1.89	2.61	-	-
25.94	Tetradecane	C ₁₄ H ₃₀	Alkanes	-	1.65	0.19	1.72	-	0.75
25.96	Hexadecane	C ₁₆ H ₃₄	Alkanes	1.45	-	0.84	-	1.37	-
25.96	Hexadecane	C ₁₆ H ₃₄	Alkanes	13.42	-	10.95	15.56	4.45	-
27.03	Tridecylcyclohexane	C ₁₉ H ₃₈	Cycloalkanes	-	-	-	-	0.06	-
28.10	Methyl isoheptadecanoate	C ₁₈ H ₃₆ O ₂	Esters	-	-	0.85	1.19	0.31	0.52
28.83	-	-	Unknown	-	-	-	-	0.33	-
28.85	3-Methylheptadecane	C ₁₈ H ₃₈	Alkanes	2.78	0.23	2.39	2.89	1.46	-
29.19	1,37-Octatriacontadiene	C ₃₈ H ₇₄	Alkenes	-	-	-	0.54	-	0.33
29.28	Octadecane	C ₁₈ H ₃₈	Alkanes	-	0.46	0.35	-	1.17	0.76
29.30	Octadecane	C ₁₈ H ₃₈	Alkanes	7.05	-	1.70	-	-	-
29.47	Methyl oleate	C ₁₉ H ₃₆ O ₂	Esters	-	-	0.42	0.71	0.20	0.72
31.02	Methyl linolelaidate	C ₁₉ H ₃₄ O ₂	Esters	-	0.47	-	-	-	-
31.91	Pentatriacontane	C ₃₅ H ₇₂	Alkanes	0.96	-	0.84	0.59	0.63	-

32.31	Eicosane	C ₂₀ H ₄₂	Alkanes	-	-	-	-	-	0.18
32.42	Methyl oleate	C ₁₉ H ₃₆ O ₂	Esters	0.19	-	-	-	-	-
32.51	Methyl oleate	C ₁₉ H ₃₆ O ₂	Esters	-	-	-	0.87	1.11	0.86
32.54	Methyl (7E)-7-hexadecenoate	C ₁₇ H ₃₂ O ₂	Esters	-	-	0.56	-	-	-
32.71	Methyl heptadecanoate	C ₁₈ H ₃₆ O ₂	Esters	-	-	2.74	0.25	-	0.20
32.79	-	-	Unkown	-	-	0.40	0.28	-	0.28
33.78	Methyl oleate	C ₁₉ H ₃₆ O ₂	Esters	-	0.59	2.39	-	0.59	-
33.80	Methyl-trans-oleate	C ₁₉ H ₃₆ O ₂	Esters	0.59	-	-	-	-	-
35.06	-	-	Unknown	-	-	-	-	0.14	0.18
35.06	-	-	Unknowns	0.10	-	-	-	-	0.08
35.25	-	-	Unkown	-	-	0.29	1.46	0.17	-
35.31	Methyl oleate	C ₁₉ H ₃₆ O ₂	Esters	-	0.40	-	-	-	-
35.49	Bis(2-ethylhexyl) maleate	C ₂₀ H ₃₆ O ₄	Esters	-	0.37	-	-	0.54	-
	Fumaric acid, bis(2-								
35.50	ethylhexyl) ester	C ₂₀ H ₃₆ O ₄	Esters	0.15	-	-	-	-	-
40.99	Eicosane	C ₂₀ H ₄₂	Alkanes	-	-	-	-	0.51	-
42.04	8-Hexylpentadecane	C ₂₁ H ₄₄	Alkanes	-	-	-	-	0.43	-