The performance of a limited aerated anaerobic membrane bioreactor for treating synthetic black water spiked with common Indian antibiotics

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Abstract

With increasing pressure on natural water resources, wastewater is gradually being considered as a potential source for potable water. Current WWTPs are designed for the removal of parameters like solids, nutrients, organic matter, and pathogens. For achieving a high-quality effluent, that enables reuse, it is important to also address the removal of micropollutants, particularly antibiotics, from the wastewater. Due to these antibiotics, antibiotic resistance spreads among the microorganisms and increases through various mechanisms. Antibiotics of sulfamethoxazole (SMX), trimethoprim (TMP), ciprofloxacin (CIP), and ampicillin (AMP) are known to be found abundantly in natural waters all across the globe. The abilities of an anaerobic membrane bioreactor (AnMBR) of maintaining high SRTs with low biomass losses help in treating wastewater containing antibiotics. A recently developed technique of adding limited aeration to AnMBR has the potential of removing recalcitrant antibiotics by improving the performance of the reactor. Hence, this research aims to study the removal mechanisms of the antibiotics (SMX, TMP) and the persistence of corresponding antibiotic resistance in AnMBR, followed by the effect of the antibiotics on the performance of the AnMBR. In addition, antibiotics CIP and AMP were tested via anaerobic batch tests to investigate the effect of the limited aeration on their removal.

After adding the antibiotics SMX and TMP to the reactor, no significant difference in COD and nutrients removal was observed. The biogas production was reduced slightly after the addition of SMX 150 µg/L initially, however, it increased back to the original state after few days. Total removal of SMX and TMP was 86% and 97% respectively in the reactor. Results showed that 85% of SMX and 94% of TMP were removed through biodegradation/biotransformation and 14% of SMX and only 3% of TMP were discharged through the effluent. From the adsorption batch tests conducted, it was observed that the linear adsorption isotherm fits well for TMP. With the increase in temperature, the adsorption potential of TMP was reduced with a K_d value of 1.234 L/g at 10˚C and 0.513 L/g at 37˚C. The removal of SMX was low through adsorption and high due to degradation and follows the first-order rate kinetics with a half-life of 1.71 days. After two weeks of SMX addition to the reactor, almost all the bacteria present in the effluent gained resistance either to TMP or SMX or both. Of all the ARGs measured in this study, the genes responsible for the resistance development were *sul1* and *sul2*. The addition of antibiotics increased the presence of ARGs in the system. The correlation between the presence of *sul1* and TMP resistant bacteria, and *sul1* and SMX resistant bacteria was 0.91-0.93, indicating that the gene *sul1* might be involved in multidrug resistance. ARGs *sul1, sul2, and dfrA1* were removed respectively by 3.2 log, 3.6 log, and 7.3 log units by the membrane. In addition, the class 1 integrons and 16s rRNA were removed by 3 log and 3.2 log units respectively. Removal of CIP and AMP was found to be high with values of 82% and 84% respectively in limited aeration assisted anaerobic batch tests. The removal efficiencies of all antibiotics were more than 80% and independent of their initial concentrations in the selected range. The increase in the removal of CIP and AMP in comparison to literature, points to a relation with the added limited aeration. Nevertheless, more studies need to be performed to establish this.

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Nomenclature

WWTP	Wastewater treatment plant
WW	wastewater
AnMBR	Anaerobic membrane bioreactor
COD	Chemical oxygen demand
SRT	Solids retention time
HRT	Hydraulic retention time
AB	Antibiotic
SMX	Sulfamethoxazole
TMP	Trimethoprim
CIP	Ciprofloxacin
AMP	Ampicillin
ARB	Antibiotic resistant bacteria
ARG	Antibiotic resistant gene
MGE	Mobile genetic elements
HGT	Horizontal gene transfer
VFA	Volatile fatty acids
K_{OW}	Octanol-water partition coefficient
TS	Total solids
TSS	Total suspended solids
VS	Volatile solids
VSS	Volatile suspended solids
CSTR	Continuous stirred tank reactor
BD	Biodegradation
BMP	Biomethane potential
GC	Gas chromatography
LC-MS	Liquid chromatography coupled with mass spectrometry
DNA	Deoxyribonucleic acid

RNA	Ribonucleic acid
qPCR	quantitative polymerase chain reaction
IF	Intermittent feeding
PC	Positive control
SMX RB	SMX resistant bacteria
TMP RB	TMP resistant bacteria

1. Introduction

1.1. Background and motivation

Due to the rapid population growth and increase in industrial and agricultural activity, concerns are raising regarding the water stress in both water-scarce countries and developing countries (Radjenović et al., 2009). According to WHO, globally about 850 million people have no access to drinking water (WHO/UNICEF, 2017). The estimation of the world population by 2050 is 9.4-10.2 billion people with the expected rise in water demands of the industrial sector by 400% (Torretta et al., 2020). Hence, instead of overexploitation of the natural resources present, alternative solutions should be implemented. For sustainable water management practice, domestic, urban, or industrial wastewaters can be regarded as an interesting source for reuse. This can provide various social, economic, and environmental benefits (Raschid-Sally and van Rooijen, 2010) and improve the state of the environment, by reducing the consumption of natural resources and decreasing the pollutants released from the WWTPs into the water bodies.

In the course of the past few decades, the technologies involved in the treatment of wastewater (WW) have improved. Also, in low-middle income countries, the number of wastewater treatment plants (WWTPs) has increased (Libhaber and Orozco-Jaramillo, 2012). According to the Central Pollution Control Board of India, around 522 sewage treatment plants are in working condition that can treat 38% of the WW generated (CPCB, 2015). Due to the rapid population growth in India, it was estimated that the water availability per capita per year will reduce from 1588 m^3 currently to 1191 m^3 by the year 2050 (India-WRIS wiki, 2019). Countries with less than 1700 m^3 water per capita per year are regarded as water-stressed. Hence, the reuse from wastewater treatment plants might provide an attractive solution by tackling both the problems of water scarcity and the pollution of water bodies due to the dumping of wastewater directly without any proper treatment.

LOTUS^{HR} (Local Treatment of Urban Sewage for Healthy Reuse) project that is being implemented in Barapullah drain, New Delhi, India, is a Dutch-Indian collaboration. This project has objectives of recovery of water, energy, and nutrients from the wastewater produced in the megacities by developing a novel holistic wastewater management technique (lotushr.org). The technologies used in this project are chosen in such a way that they are compact, robust, and cost-effective. This project has sewage pre-treatment and energy recovery as one of its three research lines. Anaerobic digestion in combination with membrane technology called anaerobic membrane bioreactor (AnMBR) is being used due to its potential for the recovery of nutrients, and energy through biogas production (lotushr.org). This research will contribute to the LOTUS^{HR} project by studying the removal of antibiotics and antibiotic resistance for the safe reuse of water, through AnMBR assisted with limited aeration.

1.2. Problem statement and Research objective

For the safe reuse of water, effluent quality from the wastewater treatment plants is an important aspect. Conventional WWTPs that receive municipal wastewater, wastewater from hospitals, industries, agricultural streams, and runoff are designed generally for the solids, nutrients, organic matter, pathogens removal, but do not consider the micropollutants and persistent xenobiotic compounds. The micropollutants removal of pharmaceuticals and personal care products (PPCPs) from wastewater is only adopted by a few countries as part of their water protection legislation (Weissbrodt et al., 2009). The presence of these micropollutants is considered an issue of emerging concern. As their removal was not considered in the design of WWTPs, PPCPs get discharged into the groundwater, rivers, oceans, and soil with the effluent of the WWTPs (Balakrishna et al., 2017).

Pharmaceuticals like antibiotics, analgesics, endocrine disruptors, β-blockers, etc. have been detected in the WWTP's effluent (Mutiyar and Mittal, 2013). As antibiotics are an important component of human and veterinary medicines, their consumption is increasing daily, leading to their occurrence in the WW of all sectors. India is in the top five producers of pharmaceuticals which produced more than 2300 Mt of antibiotics in 2006-2007 (Balakrishna et al., 2017). Among these, around 85% of the antibiotics are reportedly consumed by the domestic markets. Around 90% of these consumed antibiotics are excreted without any change (Mutiyar and Mittal, 2014).

The persistence of these unwanted antibiotics in WW increased antibiotic resistance in the microbial communities. WWTPs are considered as the major points of antibiotic resistance release into the environment. The non-resistant bacteria can gain the resistance mechanisms from the antibiotic resistance bacteria (ARB) via an exchange of mobile genetic elements (MGE) like plasmids, integrons, and transposons, that contains antibiotic resistance genes (ARG) (Blair et al., 2015). The high availability of the microbes in WWTPs promotes the transfer of antibiotic resistance via vertical and horizontal gene transfer in the presence of the antibiotics (Zarei-Baygi et al., 2019). Previous research shows that the higher solids retention time of the WWTPs resulted in the increased abundance of ARGs in conventional and membrane-based activated sludge systems (Zhang et al., 2018; Xia et al., 2012).

In Europe, per year approximately 25000 deaths, and in the US around 23000 deaths were due to ARB. Besides, around $1.5 and $1 billion were attributed to annual healthcare costs in Europe and the US respectively (Walker and Fowler, 2011; CDC, 2013). In India, approximately 56000 newborns die due to sepsis, which is caused by organisms resistant to first-line antibiotics (Laxminarayan et al., 2013). By the year 2050, in India, a cumulative of 2 million deaths are predicted to occur due to antimicrobial resistance (Dixit et al., 2019). Antibiotic resistance is one of the most critical health risks in humans (World Health Organisation, 2018). Hence, there is an urgency to look into the removal of antibiotics and antibiotic resistance by developing new and efficient methods for their removal.

Removal of the emerging pollutants occurs through various biological and non-biological processes. The removal through non-biological processes can be achieved by ozonation, filtration processes like reverse osmosis, nanofiltration, and adsorption using activated carbon. But these processes are expensive with high capital and operational costs (Buarque et al., 2019). The advancing technology of AnMBR has fewer energy requirements while producing less amount of solids compared to the aerated systems. Due to its ability to maintain high solids retention time independent of hydraulic retention time, it occupies less area and produces a very high effluent quality at a range of operational temperatures (Harb and Hong, 2017). Also, a recent technique of adding limited aeration to the anaerobic digestion process has been shown to enhance the removal of COD along with biogas production and degradation of recalcitrant compounds in the anaerobic systems (Buarque et al., 2019; Q. Chen et al., 2020; Nguyen and Khanal, 2018).

AnMBR is effective in treating pharmaceutical wastewater containing various antibiotics, and other micropollutants (Ji et al., 2020). In membrane bioreactors, besides biological removal, the membrane can retain the microorganisms and remove the larger mobile genetic elements by physical processes. The research by Kappell et al. (2018) using the primary clarifier effluent, indicated a 3.5-log reduction of ARGs *sul1*, *ermB*, and *tetO* in AnMBR. In a study by Zarei-Baygi et al. (2019), it was detected that the addition of antibiotics can change the abundance of related and unrelated antibiotic resistant genes in AnMBR. However, only a few studies investigated the removal of antibiotics and their effect on the corresponding ARB and ARGs by adding them to the feed of AnMBR (Kappell et al., 2018; Zarei-Baygi et al., 2020, 2019). Hence, **the main objective of this study is to verify the performance of limited aeration assisted AnMBR for treating synthetic black water spiked with common Indian antibiotics**.

Due to the high abundance of antibiotics and their corresponding genes, sulfamethoxazole (SMX), trimethoprim (TMP), ciprofloxacin (CIP), and ampicillin (AMP) were selected for their analysis in this research. Out of these antibiotics, SMX and TMP will be directly added to the AnMBR system for the analysis of the removal and fate of antibiotics, and antibiotic resistance. In addition, as the antibiotics CIP and AMP are known to be removed majorly in aerobic conditions, these are selected to observe the effect of limited aeration on their removal through batch tests.

1.3. Thesis outline

Chapter 1 starts with a brief introduction to the research topic, followed by the motivation of studying antibiotics and antibiotic resistance. **Chapter 2** provides the literature review relevant to the present research which includes the prevalence of antibiotics, antibiotic resistance, and their removal through physical and biological removal mechanisms. In addition, the importance of AnMBR and the introduction of the limited aeration to the reactor is presented. Along with this, the research gaps, hypothesis, research questions, and the scope of the study are given in this chapter. **Chapter 3** presents the materials and methodologies that were used in this study. It includes the description of the lab-scale AnMBR and the batch tests conducted in this research. Methods used to quantify the performance of reactor, antibiotics, bacteria, and genes are presented

here. **Chapter 4** provides the major results and discussions of this research. It presents the impact of the addition of antibiotics on reactor performance. It also consists of the results of removal and fate of the antibiotics by presenting the removal via different mechanisms. The results of antibiotic resistant bacteria and genes quantification are also discussed here. This chapter also focuses on the removal of CIP and AMP via anaerobic degradation batch tests assisted with limited aeration. **Chapter 5** provides the key conclusions of the research. **Chapter 6** presents a few recommendations for future studies.

2. Literature Review

2.1. Prevalence of Antibiotics

The consumption of PPCPs like disinfectants, drugs, body lotions, etc. has been increasing recently. Because of their harmful effects on marine life and public health, in recent years, these PPCPs have been receiving increasing attention (Singh et al., 2019). Among these pollutants, antibiotics, that are used in preventing bacterial infections, have raised more concerns due to their direct toxicity to aquatic organisms. As antibiotics also aid in developing the antibiotic resistance among pathogens, they are considered to be harmful.

Between 10-90% of the antibiotics consumed are finally excreted into the environment in their original form, with remaining as their metabolites or conjugates (Balakrishna et al., 2017). From the production in the industries to their consumption by humans and animals, these antibiotics reach the WWTPs and will finally be discharged in raw or treated form into the surface water and ground water bodies like rivers, oceans, etc. which is further used in drinking water purposes. They are released into the environment in several paths as shown in Figure 1.

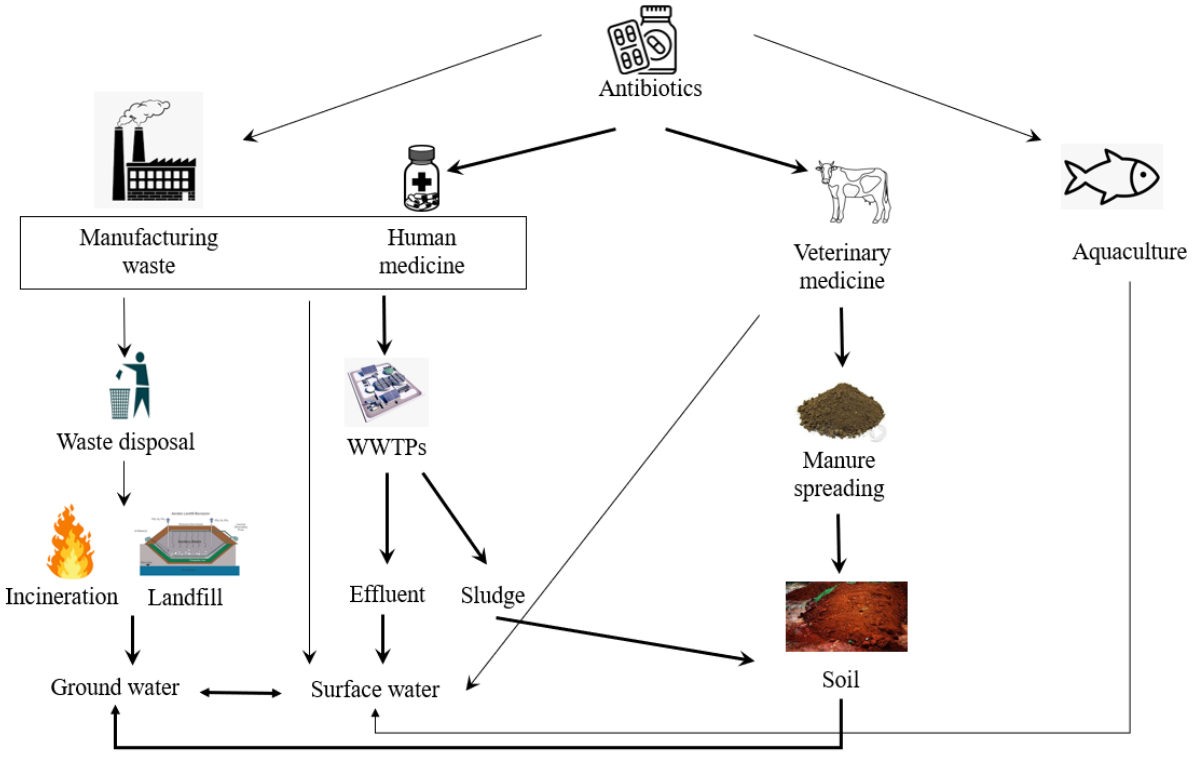

Figure 1 Pathways of how antibiotics are released into the environment. They reach the surface water, ground water in different ways, and the main paths starting from human and veterinary medicine consumption are shown. The arrow thickness indicates the relative importance of the pathway release of antibiotics

Antibiotics of several classes such as sulfonamides (e.g. sulfathiazole, sulfamethoxazole), trimethoprim, β-lactams (e.g. ampicillin), quinolones (e.g. ciprofloxacin, levofloxacin), glycopeptides, and tetracyclines (e.g. doxycycline) are being used worldwide for the treatment of a wide range of diseases due to various bacterial infections. According to Van Boeckel et al. (2015, 2014), antibiotic consumption in the animals of livestock, pigs, etc. is higher than human consumption. Despite its high usage, the quantitative data of antibiotics is not sufficiently available. Among the available antibiotics, a few like sulfamethoxazole (SMX), trimethoprim (TMP), ciprofloxacin (CIP), ofloxacin (OFX), tetracycline (TET), ampicillin (AMP), erythromycin (ERY) are found in significantly high concentrations all over the world, as these are the highly used antibiotics in human and veterinary medications (Van Boeckel et al., 2015, 2014).

Excessive utilization of antibiotics can be seen majorly in most Asian countries, leading to the ultimate distribution of antibiotics into aquatic bodies, and subsequent resistance development. As India is in the top five producers of pharmaceuticals in the world, the discharge of antibiotics from the industrial pathway into rivers is high in India. The highest antibiotic concentrations reported in Asia were from the Isakavagu-Nakkavagu streams in India. The reported concentrations were 2500 µg/L of ciprofloxacin followed by ofloxacin with conc. of 10 µg/L, norfloxacin with conc. of 4.7 µg/L and trimethoprim with a conc. of 4 µg/L (Fick et al., 2009). Due to the high concentrations of antibiotics in the surface waters, a wide range of quinolone and β-lactam resistant antibiotic resistant genes has been reported in Indian rivers. A broad range of antibiotic concentrations was observed in the water bodies from different regions of the world. Some of the available concentrations of widely used antibiotics are shown in Table 1. After treatment, the concentration of antibiotics in drinking water is found to be less than 50 ng/L (Maycock and Watts, 2011). The concentrations of antibiotics above this range are considered to be potentially harmful. The concentrations of SMX in rivers from China like Wangyang River, Hai River were considerably high with a value of 4.8 µg/L. High values of TMP concentrations were also recorded in Isakavagu-Nakkavagu (India) and Ravi River (China) with 4 µg/L and 1.1 µg/L respectively.

As trimethoprim-sulfamethoxazole is an efficient and inexpensive antibiotic prescription available, it has been used widely for many years to treat bacterial infections. In European rivers, among all the antibiotics, SMX, TMP, CIP, and ERY were found in abundance (Johnson et al., 2015). The maximum concentrations of SMX found were in Portugal with a conc. of 8.7 µg/L, followed by Italy and France with concentrations of 6.5 µg/L and 1.4 µg/L respectively. Similarly, the next abundantly found ciprofloxacin has concentrations of 3.7 µg/L, 1.4 µg/L respectively in Italy and Portugal.

The values mentioned in Table 1 are of the antibiotic concentrations found in the rivers. However, the concentrations reaching the wastewater treatment plants are found in higher values than those mentioned in Table 1. The maximum concentration of ciprofloxacin in the water treatment plant, PETL (Patancheru Enviro Tech Limited) receiving the wastewater from 90 drug manufacturers was the highest ever reported concentration of the antibiotics with a value of 14000 µg/L of ciprofloxacin (Fick et al., 2009). Similarly, the maximum concentrations of SMX, CIP reaching

the WWTPs of the Netherlands are 0.3 µg/L, and 1.4 µg/L respectively. Due to such high antibiotic concentrations in WWTPs, they act as a hotspot for the growth and spreading of ARGs.

Table 1 Concentrations of antibiotics in rivers from around the world

Continent/Country	Antibiotic concentration (µg/L)				Reference
	SMX	CIP	TMP	AMP	
Asia					
India		2500	4.00		(Fick et al., 2009)
India		1.44			(Mutiyar and Mittal, 2014)
Hong Kong	0.03	0.03			(Deng et al., 2018)
Hong Kong		0.72		0.40	(Li et al., 2009)
China	0.08		0.08		(Xu et al., 2018)
China	4.87	0.55	1.13		(Chen et al., 2018)
China	0.07				(Wang et al., 2017)
Iran		0.02			(Mirzaei et al., 2019)
Taiwan	0.04				(Hsu et al., 2014)
America					
Canada		0.6			(Guerra et al., 2014)
USA	0.015				(Picó and Andreu, 2007)
Europe					
Holland	0.10		0.18		(Sabri et al., 2020)
Portugal	8.72	1.40			(Santos et al., 2013)
Germany	0.48		0.20		(Hirsch et al., 1999)
France	1.44	0.14	0.25		(Tuc Dinh et al., 2011)
Spain	0.07	0.07	0.93		(Rodriguez-Mozaz et al., 2015)
Italy	6.50	3.70			(Verlicchi et al., 2012)
Australia	2.00	1.30	0.15		(Watkinson et al., 2009)

2.2. Removal of selected antibiotics in WWTPs

In this research, based on the abundance of antibiotics and their corresponding ARG in different parts of the world, four antibiotics TMP, SMX, AMP, and CIP were chosen to study for their removal. The important properties of these antibiotics are shown in Table 2.

Table 2 Properties of selected antibiotics

Antibiotic	Molecular formula	Log K_{ow}	Charge (pH 7-9)	pKa	Henry's constant ($atm.m^3/mol$)
TMP	$C_{14}H_{18}N_4O_3$	1.26	+ve	7.12	2.14E-14
SMX	$C_{10}H_{11}N_3O_3S$	0.89	-ve	5.12	6.40E-13
AMP	$C_{16}H_{19}N_3O_4S$	1.35	+ve	2.50	2.40E-17
CIP	$C_{17}H_{18}N_3O_3F$	0.28	+ve	6.09	5.09E-19

2.2.1. General mechanisms of antibiotics removal

The removal of antibiotics might happen by two processes: biotic, and abiotic/non-biotic processes. The biotic process mainly includes the degradation of antibiotics by bacteria and fungi. Abiotic processes mainly consist of physical or chemical processes like sorption, hydrolysis, photolysis, volatilization, etc. However, as the wastewater treatment plants have little exposure to light, the photolysis mechanism may not be the path of antibiotics removal. Hydrolysis can be a removal pathway for some antibiotics. However, as the molecular weights of the selected antibiotics are high and they do not contain favorable functional groups, hydrolysis can be negligible (Liu et al., 2010; Thi Mai, 2018). Volatilization is the process where the dissolved antibiotics get transferred to the gaseous form. This depends on Henry's constant of the compounds and the operational conditions. However, according to Namkung and Rittmann (1987) volatilization can only be considered as a removal pathway if Henry's law constant of the compound is above 1.0E-3 atm-m^3/mol. Hence, the main antibiotics removal mechanisms in the WWTPs are considered as sorption and biodegradation (Michael et al., 2013).

Antibiotics can be removed from the aqueous phase onto the solid phase by sorption, complex formation with metal ions, ion exchange, and polar hydrophilic interactions (Díaz-Cruz et al., 2003). The hydrophobic antibiotics can be removed easily via sorption onto sludge, compared to the hydrophilic antibiotics, because of their greater affinity to solids. This tendency of antibiotics to adsorb on sludge can be evaluated by the octanol-water partition coefficient (K_{OW}). According to Rogers (1996), the organic contaminants have a low sorption potential if log K_{OW} < 2.5. The organic contaminants with 2.5<log K_{OW}<4 have medium sorption potential, and the contaminant with log K_{OW} > 4 have a high sorption potential. A few antibiotics that have low sorption potential are tetracyclines, sulfonamides, aminoglycosides. Antibiotics with medium potential are β-lactams, macrolides, and the antibiotics with higher potential are glycopeptides (Michael et al., 2013).

The prediction of sorption of antibiotics on sludge based on log K_{OW} values can be done mainly for the non-polar compounds. The prediction of sorption on polar antibiotics might not be correct often. The use of log K_{OW} values also leads to an underestimation of sorption in a few antibiotics like fluoroquinolones, tetracyclines (Golet et al., 2003; Kim et al., 2005). For example, ciprofloxacin, a fluoroquinolone class antibiotic, has a log K_{OW} value of 0.28, yet it is 80% sorbed onto sludge, indicating that the main removal pathway is sorption. However, as the sorption

process occurs in parallel to the biodegradation, it is very difficult to analyze the removal, just via sorption. In several studies on the removal of SMX, it was observed that the removal due to the sorption was below 10% (Göbel et al., 2007; Michael et al., 2013; Yang et al., 2005).

The removal of antibiotics in the WWTPs depend on many operational factors such as ORP, hydraulic retention time (HRT), SRT, pH, suspended solids loading, temperature, food-microorganism ratio (F/M), and dissolved oxygen (Drewes, 2007; Kovalova et al., 2012; Michael et al., 2013).

The removal of antibiotics through biodegradation can be increased with higher SRTs. As the SRT is related to the microbial growth rate, higher SRTs assist the growth of slowly growing bacteria, which leads to diverse enzymes that assist in degrading the antibiotics (Göbel et al., 2007; Le-Minh et al., 2010). A higher SRT combined with a reduced F/M ratio was found to favor the removal of the antibiotics via degradation. The higher SRTs can be reached in the MBRs by retaining sludge in the reactor with the help of the membranes that can separate the solid-liquid phase. Mostly used membranes in the MBRs are microfiltration and ultrafiltration membranes. The retention of these antibiotics on the membrane is considered to be negligible (Radjenović et al., 2009; Tadkaew et al., 2010). The removal of selected antibiotics reported in the literature is summarized below.

2.2.2. Antibiotics removal in aerobic conditions

In the previous study by Li and Zhang (2010), the removal of 11 antibiotics from different classes via biodegradation, adsorption, and volatilization in the conventional activated sludge (CAS) process was verified. The removal of antibiotics through volatilization was observed to be negligible. As shown in Figure 2, for antibiotics TMP, CIP, and AMP, adsorption was found to be the main removal mechanism, whereas for SMX it was biodegradation. The removal efficiencies

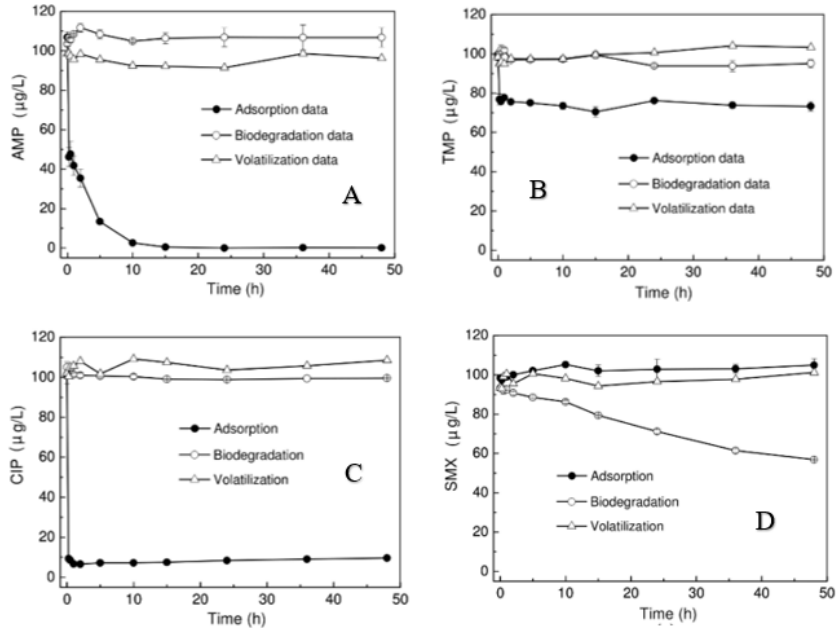

Figure 2 Removal of selected antibiotics via different pathways in CAS process (Li and Zhang, 2010)

of CIP, AMP were found to be high in the aerobic activated sludge process, 85% and 98% respectively. Whereas the removal of TMP and SMX was less, 26.4% and 39.1% respectively.

Göbel et al. (2007) observed the removal of SMX and TMP in several municipal WWTPs. The SMX removal was independent of SRT, with a value of ~80% in the MBR system, compared to ~60% in the CAS system. The removal of TMP was increased from 50% to 90% with an SRT increase from 23 days to 70 days indicating a correlation with decreased loading of the substrate. The combination of high SRT with reduced sludge loading might have caused the increase in biodiversity of the biomass, influencing the removal of antibiotics undergoing co-metabolism (Göbel et al., 2007).

In aerobic MBR, CIP removal was observed to be in the range of 60-90%, in which a flat-sheet membrane reactor showed higher removal efficiencies compared to a hollow-fiber membrane (Nguyen et al., 2017). Hamjinda et al. (2017) observed only 58% removal of CIP in a two-stage (2S) MBR with an anoxic reactor, aerobic MBR due to its recalcitrant nature. However, a 3S MBR with a pretreatment step improved the removal of CIP to 90%. The removal of AMP in the airlift biofilm reactor was observed to be high with 90-98%, out of which around 40% was due to biodegradation and the remaining was found to be due to adsorption (Shen et al., 2010).

2.2.3. Antibiotics removal in anaerobic conditions

The removal efficiencies of TMP and SMX are expected to be higher with a value of ~85% at the lower redox potential condition. The SMX and TMP are readily biodegraded under anaerobic conditions because of their chemical structures. The amide group present in the SMX makes its degradation difficult in aerobic conditions. However, it can be transformed by reductive reactions due to the electron-withdrawing group like sulfonyl in the anaerobic conditions. Similarly, in the case of TMP, the substituted pyrimidine group can be readily biotransformed in anaerobic conditions (Alvarino et al., 2018, 2016).

As the antibiotic sulfamethoxazole has a negative charge and low sorption capacity at neutral pH, the main mode of removal for SMX is expected to be biodegradation. The removal efficiencies of sulfamethoxazole and trimethoprim in the AnMBR system fed with synthetic black water at a concentration of 1.5 µg/L, was observed to be 95.2%, and 40% respectively by Monsalvo et al. (2014). On the other hand, Wijekoon et al. (2015) observed a 98% removal of trimethoprim in AnMBR, most of which was removed via adsorption. Wei et al. (2019) examined the removal of SMX via an AnMBR system with varying influent concentrations of 10-100000 µg/L. The removal of SMX at all concentrations was observed to be due to biodegradation, with a value of more than 88%. Hence, using the AnMBR systems, a constant high removal of SMX and TMP was observed in the literature, which makes the AnMBR a promising technology for treating the municipal wastewater containing the selected antibiotics.

In a study conducted by Thi Mai (2018), it was shown that CIP removal of 50-76% can be observed in AnMBR at lower concentrations. In AnMBR, the removal of AMP was found to be very low,

with a removal efficiency of 22-43% (Huang et al., 2018a) compared to 90-98% in airlift biofilm reactors (Shen et al., 2010). Only a few studies were found in the literature regarding the removal of CIP and AMP in anaerobic conditions.

2.3. Removal of antibiotic resistant bacteria and genes in WWTPs

The capacity of microorganisms to reduce the efficacy of antibiotics by resisting and escaping from their effects is known as antibiotic resistance (Singh et al., 2019). The high concentrations of microorganisms in the wastewater treatment systems promote this antibiotic resistance spreading through horizontal gene transfer (HGT) and clonal expansion if they are exposed to lethal levels of antibiotics. Through horizontal gene transfer, the dissemination of antibiotic resistance takes place. While the clonal expansion works on the amplification of the genes within the individual hosts. If the HGT occurs frequently, it would broadly distribute the resistant genes that result in many gene combinations. In such cases, regionally independent gene distributions can be expected. On the other hand, if the clonal expansions occur frequently, the region-dependent genes with high antibiotic resistance levels and unique gene combinations are expected (Blahna et al., 2006).

According to Pärnänen et al. (2019), the most prevalent antibiotic resistant genes in European countries of selected antibiotics are *sul1*, *sul2*, *dhfr1* (sulfonamides), *qnrS*, *qnrC*, *qnrD* (quinolones), bla_{GES}, bla_{OXA}, bla_{VEB} (β-lactams), *dfrA1*, *dfrA17* (trimethoprim). A few studies have reported the positive correlation of the concentration of the antibiotics with the abundance of the corresponding resistance genes (Figure 3). The surveillance in Europe showed that their WWTPs have higher ARGs in the high antibiotic consumption countries like Spain, Portugal, Ireland. Whereas the ARGs are found in lower concentrations in the countries like Norway, Finland where antibiotic consumption is relatively low (Pärnänen et al., 2019).

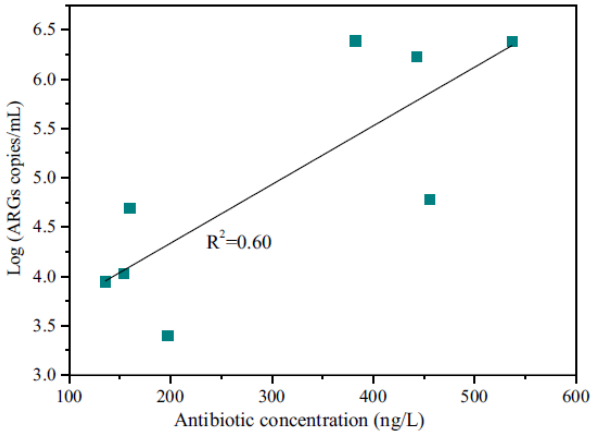

Figure 3 Positive correlation between average concentration of antibiotics (macrolides, sulfonamides, quinolones and tetracyclines) and the abundance of corresponding ARGs (erm, sul, qnr and tet) in influent and effluent of WWTPs (Wang et al., 2020)

In the biological treatment processes treating antibiotics containing wastewater, the major concern is that the ARGs and ARB might grow and multiply with time. However, it was observed that around 2 logs reduction in ARGs and ARB was found in WWTPs. On the other hand, still, ~2.9-4.6 logs of ARG, 2.3-4.5 logs of ARB are reported in the effluent of WWTPs (Wang et al., 2020).

The discharge of this effluent into the environment causes huge risks for the ecology. The reuse of such effluent is also considered dangerous. According to the quantitative microbial risk assessment study conducted by Al-Jassim et al. (2015), it was observed that the effluent of WWTPs can be allowed to reuse for irrigation purposes only after applying the chlorination step as disinfection. However according to Hong et al. (2018), to reuse the effluent for non-potable purposes, micro, ultra or nanofiltration processes are sufficient. To reuse the water for potable purposes, the advanced disinfection treatment steps should be adapted. Recently a few studies investigated the removal capacity of AnMBR regarding ARGs and ARB. The research by Kappell et al. (2018) using the primary clarifier effluent, indicated a 3.5-log reduction of ARGs *sul1, ermB, and tetO* in AnMBR. Another work by Cheng and Hong (2017), showed that the subcritical membrane fouling in AnMBR increased the removal of ARGs and ARB significantly. This indicates that the membrane biofilms might play an important role in the removal of antibiotic resistance. In this research, the ultrafiltration membrane associated with the anaerobic digestion reactor was applied and studied for antibiotic resistance.

2.4. Performance of anaerobic digestion

Anaerobic digestion (AD) includes the biodegradation of organic matter (OM) in the absence of oxygen, into methane and carbon dioxide. This breakdown of OM is done in various stages by several types of hydrolytic, fermentative, acetogenic, and methanogenic bacteria.

Hydrolysis

In AD, hydrolysis is the first reaction mechanism which includes the breakdown of complex compounds such as carbohydrates, proteins, and lipids into simple compounds like sugar, amino acids, and peptides respectively. Hydrolytic bacteria such as *Micrococcus, Peptococcus, Clostridium* are responsible for the degradability of complex compounds in anaerobic conditions. The hydrolysis step is regarded as a rate-limiting step for the overall process when a large amount of particulate matter is present in the feed, reducing its degradability (SCHINK, 1987).

Acidogenesis

In this step, the simple organic compounds like sugars, amino acids, etc. that were formed in the hydrolysis step, will be converted by fermentative bacteria and anaerobic oxidative bacteria, into volatile fatty acids, ketones, carbon dioxide, hydrogen, alcohols. This acidogenesis step is a very fast occurring process as the bacteria responsible for this process are fast growers with a doubling time of a minimum of 0.5 h. This fast growth leads to high acid production, leading to a significant drop in pH value (Thi Mai, 2018).

Acetogenesis

In acetogenesis, the fatty acids, alcohols produced in acidogenesis are converted to acetate, hydrogen, and carbon dioxide by the acetogenic bacteria. Butyrate and propionate are the major substrates in this step. There is a production of hydrogen in this step (Phelps and Zeikus, 1984).

Methanogenesis

The methanogenesis step produces biogas, mainly consisting of methane and carbon dioxide by the degradation of acetate or carbon dioxide and hydrogen. Based on the substrate utilized, the methanogens are divided into two major groups of aceticlastic methanogens (substrate: acetate), and hydrogenotrophic methanogens (hydrogen utilizing). The doubling times of the aceticlastic methanogens range to several days with significantly low growth rates. On the other hand, hydrogenotrophic bacteria have a double-time of 4 to 12 hours with comparatively high growth rates. Two major genera of aceticlastic methanogens are *Methanosarcina* and *Methanosaeta*. *Methanosarcina* spec. have a wide substrate spectrum with its capacity to utilize many substrates like acetate, H_2/CO_2, formate, methanol, and methylamines (Van Lier et al., 2008).

The addition of SMX in concentrations greater than 45 mg/L to the anaerobic systems was found to be lethal and causes the inactivation of aceticlastic methanogens. Hydrogenotrophic methanogens and C*lostridium sp.* were found to be dominant (Cetecioglu et al., 2016). Aceticlastic methanogens like *Methanothrix* and *Syntrophobacter* were found to be affected by the addition of 0.5-50 mg/L of CIP to the anaerobic system. Accumulation of propionate in the reactor can be found on the reduction of *Syntrophobacter* (Thi Mai, 2018). The addition of antibiotics sulfamethoxazole, ampicillin, and erythromycin at a concentration of 250 µg/L did not alter the microbial community of biomass in the AnMBR system (Zarei-Baygi et al., 2020).

Besides, the performance of anaerobic digestion can be altered with the addition of a limited amount of aeration. The limited aeration improves the rate of conversion of slowly degradable COD to readily degradable COD, due to which the reactor can achieve a stable performance without any VFA accumulation. Zhou et al. (2007) detected an increase in COD removal from 40% before aeration to 80% after limited aeration (aeration rate: 3–6 mL L^{-1} min^{-1}). It was also observed that the biogas quality was improved after limited aeration, by activating the sulfide oxidation and hydrogen sulfide removal (Mahdy et al., 2020). Hence, it can be seen that the performance of the reactor can be altered by the addition of antibiotics or limited aeration to the system.

2.5. Research gaps

As mentioned in section 2.2, even though the removal of a few antibiotics in anaerobic conditions was higher than aerobic, not all antibiotics can be removed in anaerobic conditions. A few antibiotics like CIP, AMP, favor aerobic conditions because of their recalcitrant nature. Hence, there is a need to adopt an advanced process of limited aeration by combining both anaerobic and aerobic systems, to achieve the removal of most of the antibiotics. Despite its advantages, till now just two antibiotics of SMX and TMP were investigated for their removal through limited aeration assisted anaerobic digestion process by Buarque et al. (2019) and do Nascimento et al. (2021) in a lab-scale upflow anaerobic sludge blanket reactor. In addition to studying the removal of antibiotics, it is important to study the fate and transport of the removed antibiotics in the WWTPs.

Furthermore, in the biological treatment processes treating antibiotics containing wastewater, the major concern is the dissemination of antibiotic resistance with time. The presence of antibiotic resistance in the effluent of the WWTPs is harmful and prevents the reuse of wastewater. Although AnMBR has high potential in treating wastewater, to the best of the author's knowledge, its effectiveness in removing the antibiotics and corresponding antibiotic resistance has been rarely investigated (Kappell et al., 2018; Zarei-Baygi et al., 2020, 2019).

2.6. Hypothesis and Research Questions

The main research question based on the discussion made in previous sections was formulated as following to tackle the problem effectively**.**

What is the performance of a limited aeration assisted AnMBR for treating synthetic black water spiked with common Indian antibiotics?

The following research objectives and their corresponding hypothesis were formulated to answer the main question:

Objective 1: To verify the effect of the addition of antibiotics SMX and TMP on the performance of the AnMBR used in this study in terms of COD, nutrients removal, and biogas production

Hypothesis 1: The a*ddition of antibiotics to the reactor reduces the performance of AnMBR by accumulating the VFAs and affecting the COD removal efficiency, biogas production, and nutrients by more than 10%.*

Objective 2: To evaluate the effect of limited aerated AnMBR on the antibiotics and antibiotic resistance

- Analysis of the removal efficiencies and fate of the antibiotics SMX and TMP
    - Assessing the removal of antibiotics through adsorption
    - Assessing the removal of antibiotics through biodegradation
- Analysis of the ARB and ARGs corresponding to SMX and TMP in biomass and effluent of the reactor

Hypothesis 2: Removal efficiencies of antibiotics SMX and TMP in limited aeration assisted AnMBR is less than the removal observed in anaerobic conditions (~95%) but higher than that observed in aerobic conditions (~40%), with biodegradation as main removal pathway. Also, their corresponding antibiotic resistance can be removed using the membrane by more than 3 log units.

Objective 3: To study the effect of limited aeration on the removal of antibiotics CIP and AMP

Hypothesis 3: The removal of antibiotics CIP and AMP can be improved by the addition of limited aeration compared to the anaerobic conditions by at least 20%.

3. Material and Methods

3.1. Continuous Anaerobic membrane bioreactor

A lab-scale AnMBR was set up as a part of the LOTUS^{HR} project (Figure 4**)**. This AnMBR includes a glass reactor with a capacity of 7 L (working volume of 5.5 - 6 L), connected to an external, inside-out cross-flow membrane. This reactor has 7 ports in the top with different diameters, each connected to a different part of the system. There are 3 sampling points on the side of the reactor to collect sludge samples from reactor. The sludge inoculum used in this reactor was obtained from a 1 m^3 anaerobic reactor treating blackwater, NIOO, KNAW, Wageningen. At first, this AnMBR was operated at anaerobic conditions until it reached the stable condition, in which later a limited aeration (14.7 ml air/Lrec/d) was introduced in the reactor through a calibrated pump. This aeration was added to the reactor daily in 3 cycles, with each cycle of 8 hours. In these 8 hours, the reactor was aerated for four hours followed by 4 hours of resting. This conversion from feeding to resting was done automatically by a timer that sends a signal to the pump. The reactor was operated for more than 1.5 years with aeration.

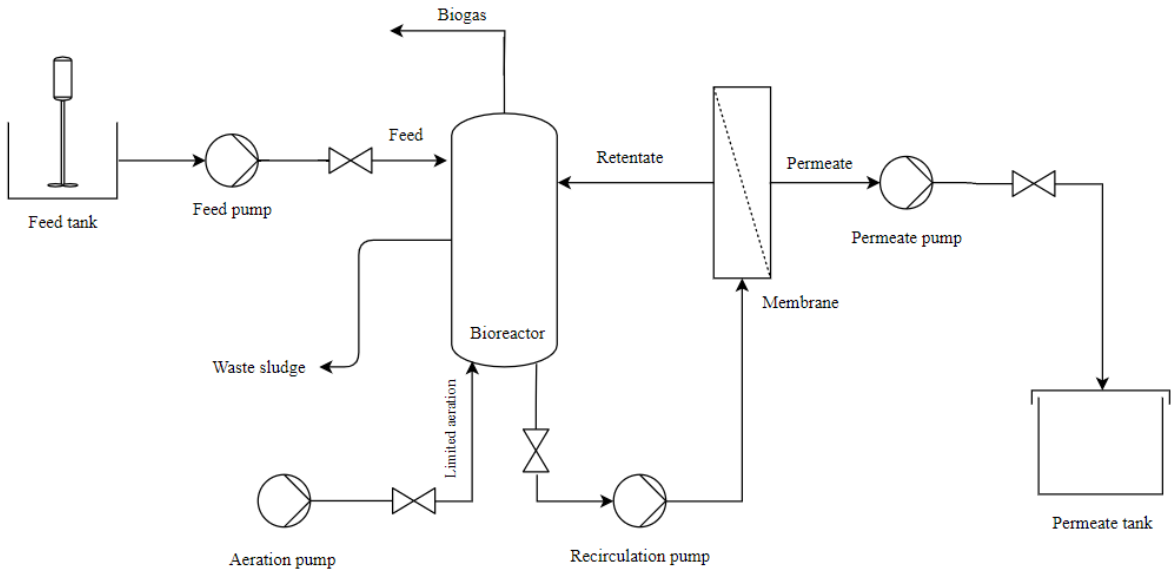

Figure 4 Piping and Instrumentation diagram of AnMBR

3.1.1. Feed preparation

The influent used in this reactor was a recipe of synthetic black water feed, that was prepared by mixing various macro and micro nutrients (Appendix A) which was adapted and altered from (Ozgun et al., 2013). This synthetic blackwater feed has an average COD of 4.9±0.6 g/L. For the preparation of feed, the ingredients mentioned in Appendix A were first added to a 2 L water and were blended using a hand blender for 2 min, to achieve a homogeneous mixture. This feed was then diluted to 12 L. This synthetic feed was prepared twice per week and stored in the fridge in a bucket equipped with a mechanical agitator to keep it homogeneous throughout the feeding time.

This feed was then pumped to the reactor through a port on top. The outflow of this CSTR was then sent to the external membrane which is a hollow tubular, helix membrane with a pore size of 30 nm. This membrane was contained in a closed glass tube, with 3 ports out of which the top port was connected to the reactor, and the bottom port was connected to a recirculation pump to recirculate the concentrate of the membrane to the reactor. The third port is the effluent outlet through which the permeate was drawn from the outside of the membrane to a permeate bucket.

3.1.2. AnMBR operational parameters

The reactor was maintained at a constant volume of 5.5 L at mesophilic conditions of 37˚C. Other operational parameters of the reactor are shown in Table 3. The inflow of the reactor was maintained at around 2.7 L/day, and the effluent flow was maintained at 2.5 L/day. The feed and permeate pumps were calibrated regularly using the sampling points of the setup. The computer interface of AnMBR was available on the software generated by CARYA Automatisering, The Netherlands (Appendix A), through which the operation of the reactor was monitored. pH, temperature, and ORP of the reactor were monitored and recorded daily using a probe. The biogas generated was measured through a gas ritter (Ritter, Germany) and monitored daily.

Table 3 Operational parameters of reactor

Parameter	Value	Unit
Feed flow	2.7	L/d
Permeate flow	2.5	L/d
Reactor volume	5.5	L
Temperature	37	˚C
Hydraulic retention time	2.1	d
Solids retention time	28	d
Sludge wasted	0.2	L/d
Flux	10	LMH
Recirculation flow	1300	L/d
Organic loading rate	2.45	gCOD/L/d

3.2. Experiments in continuous reactor

The steady-state of the reactor operation was described as consistent low COD concentration in the effluent (<60 mg/L) with stable biogas production, and high methane content in biogas (>85%) over at least two weeks of operation. Once the steady-state of the reactor was reached after the limited aeration period, two antibiotics including trimethoprim and sulfamethoxazole were added to the feed of the reactor in sequential phases.

A standard solution of 200 mg/L was prepared for trimethoprim and sulfamethoxazole using ultrapure water as a solvent for trimethoprim, and a mixture of ultrapure water with sufficient drops of sodium hydroxide for sulfamethoxazole. These stock solutions were stored at 4˚C in dark by wrapping the bottles with aluminum foil.

Phase 1: Addition of TMP

In this phase, TMP was added to the reactor in three stages of increasing concentrations of 10, 50, 150 µg/L with seven days at each concentration, which corresponds to a period of around 3 HRTs of the system. These concentrations were chosen based on the typical concentration levels found in domestic, hospital wastewaters. The highest concentration was chosen based on the maximum concentration levels found in the Indian drain water (Fick et al., 2009). Hence, after fourteen days the final concentration of 150 µg/L was added to the reactor and kept constant till the end of the study.

Phase 2: Addition of SMX

After seven days of adding the TMP concentration of 150 µg/L, SMX was added to the reactor in three stages of increasing concentrations of 10, 50, 150 µg/L with seven days at each concentration. After the end of fourteen days, 150 µg/L of SMX was added to the reactor and kept constant till the end of the study. Analysis of the removal efficiencies of antibiotics was performed in all the phases of antibiotics addition.

3.3. Experiments via batch tests

Before the addition of antibiotics to the reactor, a few batch tests were conducted to assess the impact of selected antibiotics on biomass, and their removal efficiencies. To analyze the removal of antibiotics, mainly two types of batch tests degradation and adsorption were performed.

3.3.1. Degradation tests

The main aim of the degradation batch tests was to analyze the removal of antibiotics through biodegradation by performing mass balance. Further, two sets of degradation tests were performed:

1. Batch tests with varying concentrations of antibiotics with one-time feeding
2. Batch tests with intermittent feeding at a constant concentration of 150 µg/L

These batch tests were chosen to verify the initial concentration effect, and feeding pattern effect on the removal efficiencies via biodegradation respectively.

All batch tests were conducted in serum bottles (180 mL). These bottles were fitted with a rubber stopper and sealed with an aluminum crimp using a clamper to avoid the leakage of biogas.

To maintain the solids retention time of the lab-scale limited aerated anaerobic membrane bioreactor, as mentioned in Table 3, around 200 mL of sludge was wasted from the reactor per day. Wasted sludge was collected in a 5 L container and was flushed with nitrogen every day to maintain the anaerobic condition. For the batch tests, sludge was collected for 10 days before the test and was used as inoculum. This collected sludge was analyzed for COD and solids concentration before the experiment. These properties are provided in Appendix A. In addition, as suggested by Holliger et al. (2016), the accumulated sludge was incubated at 37˚C for five days before the start of the batch tests to prevent unwanted endogenous gas production. Also, a few

blank samples with sludge and water were added to each batch test to quantify any endogenous gas production. This incubated inoculum was again subjected to solids quantification as volatilization of biomass reduces the VS content of the inoculum.

All batch tests were performed at a temperature of 37˚C similar to the reactor. The bottles were kept inside an incubator shaker with the temperature maintained at 37˚C, and constant stirring of the bottles at 160 revolutions per minute (RPM) to ensure proper mixing of the bottles. In both sets of tests, $CH_3COONa.3H_2O$ (>98%, Sigma-Aldrich, Switzerland) was used as a substrate. The amount of substrate to be used was calculated according to the ratio of VS of inoculum to substrate as 2 (Holliger et al., 2016). The required amount of micronutrients were also added to the bottles and further diluted with demineralized water to make up the total volume of bottles to 100 mL. For each condition of the tests, triplicate bottles were used. After the addition of inoculum, the bottles were flushed with nitrogen for at least 2 min to create anaerobic conditions and were closed tightly.

Biodegradation tests with one-time feeding

In this set of batch tests, the concentration of substrate calculated according to the ratio as mentioned above was added completely on the first day of the experiments. In addition to the substrate and inoculum, antibiotics ciprofloxacin and ampicillin were added to the bottles at different concentrations of 10 µg/L, 50 µg/L, and 150 µg/L. The antibiotics SMX and TMP were not tested with varying concentrations, as according to the tests performed by Khande (2020) on the sludge collected from the same reactor, it was concluded that the removal efficiencies for SMX and TMP don't depend on their concentrations. Hence to predict the removal efficiencies of SMX and TMP in the reactor, the batch tests were only performed with the final concentration that would be added to the reactor, i.e., 150 µg/L. In total, the following 4 conditions were tested in this set of biodegradation tests.

1. CIP + AMP 10 µg/L
2. CIP + AMP 50 µg/L
3. CIP + AMP 150 µg/L
4. SMX + TMP 150 µg/L

All the conditions were performed in technical triplicates. In all the conditions, the limited aeration was kept constant at 4.2 mL air/batch/d, which was similar to the aeration used in the reactor, 2.03% of VS of sludge. The aeration was provided to the bottles for the first 5 days. This test was run until the methane production varied <1% of cumulative methane production for 3 consecutive days.

Biodegradation tests with intermittent feeding

This set of the test was done to simulate the reactor conditions to a maximum extent through batch tests. The concentration of antibiotics was kept constant at 150 µg/L and with aeration of 3.8 mL

air/batch/d (2.03% of VS of sludge used). The total amount of substrate calculated according to the ratio mentioned before was divided and added to bottles in 5 days similar to the addition of aeration. After a period of two weeks, where the added acetate was fully consumed, the test was extended to 28 days (which corresponds to 1 SRT of the reactor) by continuing the addition of substrate in a similar manner. The following two conditions were tested in this set.

1. CIP + AMP 150 µg/L
2. SMX + TMP 150 µg/L

To assess the impact of the antibiotics on biogas production, standard conditions (positive controls) were also added to both the sets of biodegradation tests. These controls were prepared by the addition of substrate as only acetate, without any antibiotics addition. The pressure accumulation in the bottles was measured twice per day. Around 2 mL of biogas was collected from the bottles daily in the first 4 days of the experiment, followed by twice per week, using 2.5 mL syringes, and were analyzed for their composition using gas chromatography (section 3.4.2). As the maximum pressure the bottles could withstand was 2 bars, the bottles were depressurized daily by opening them to the atmosphere using the needles.

3.3.2. Adsorption tests

For some of the selected antibiotics, adsorption was predicted to be the main removal path due to their hydrophobic properties. Hence, adsorption batch tests were performed to analyze the removal of antibiotics via adsorption.

Adsorption tests were performed in 250 mL glass bottles. As the adsorption process is a physical mechanism, it occurs fast. In these tests, as we wanted to test for the removal of the antibiotics only via adsorption, it is important to deactivate the biomass to avoid any biodegradation in the bottles. Hence, to inhibit the biomass activity, the adsorption tests were conducted at 10˚C, for 6 h. In addition, the bottles were placed on the shaker with a speed of 160 RPM and were kept open to the atmosphere, to ensure proper mixing of the bottles, and inhibit the biomass activity further.

In addition to the experiments at 10˚C, for SMX and TMP, the adsorption tests were also performed at 37˚C to simulate the reactor conditions. This was done to get a similar removal efficiency value from batch tests, to that of the reactor. The tests at 37˚C were performed only for 2 h to avoid the possible biodegradation of antibiotics. To get the adsorption isotherms for the antibiotics SMX and TMP, the adsorption tests were performed at concentrations of 10, 50, and 150 µg/L. Also, to ensure that there was no competition due to adsorption on sludge between the antibiotics SMX and TMP, the batch tests were performed separately for these antibiotics. In addition, for CIP and AMP, one preliminary adsorption test was performed at 10˚C and 150 µg/L.

Initially, the samples were taken with an interval of 5 min for the first 45 min, followed by an interval of 15 min till 2nd hour, then the samples were taken after every 30 min till 4th hour. The last sample was taken after 6 hours. After collecting, the samples were immediately centrifuged in

the mini centrifuge, and the supernatant was filtered through a 0.20 µm syringe filter, and the samples were stored at -20˚C until further analysis.

3.4. Experimental analysis

The analytical methods used in this study are explained briefly in this section.

3.4.1. COD, solids, and nutrients

Initially, COD of influent, and effluent were analyzed every day till the reactor reached its stable conditions. Following this period, the COD was tested on every alternate day. The soluble COD of influent was measured to check the variations in particulate COD of the feed in the bucket. In addition, the COD of sludge was determined to verify the COD balance of the system.

The solids and nutrients analysis was performed once per week. Nutrients of feed, sludge, and effluent tested were ammonia, nitrate, total nitrogen, phosphate, and sulfate. COD and nutrients were quantified with *Hach Lange's* kits as mentioned in Appendix A. Solids content of the sludge were analyzed following the standard methods mentioned in APHA, 1992.

3.4.2. Volatile fatty acids and biogas composition

Volatile fatty acids (VFAs) were quantified by gas chromatography (Agilent tech 7890A, US) equipped with a capillary HP-FFAP column. The sludge sample was collected daily in 15 mL tubes and centrifuged at 10000xg for 10 min and then the supernatant was filtered through a 0.45 µm syringe filter (Whatman Spartan 30/0.45RC Rinse filter) to measure VFAs. 1.5 mL of these filtrates were collected into glass vials and were acidified by adding 10 µL of formic acid to cut the microbiological activity and reduce the pH of the prepared samples for analysis.

For the analysis of the composition of biogas, the gas samples were collected on every alternate day with 10 mL syringes in duplicates. These samples were injected into a GC (Agilent 19095P-MS6, U.S.) provided with a thermal conductivity detector.

3.4.3. Quantification of Antibiotics

The quantification of antibiotics was done using the Liquid chromatography coupled mass spectrometry (LC-MS). This technique involves liquid chromatography where the individual components were separated first followed by converting the compounds into ionized states and analysis of the ions based on their mass/charge ratio.

Preparation of antibiotic stocks

All the antibiotics of SMX, TMP, CIP, AMP were purchased from Sigma-Aldrich (>98% TLC). All the solvents used were of HPLC grade. The concentrated stocks of 200 mg/L of sulfamethoxazole, trimethoprim, ciprofloxacin, and ampicillin were prepared as follows:

1. SMX stock solution was prepared by mixing 200 mg SMX in 1 L ultrapure water with a few drops of sodium hydroxide till it dissolved.

2. TMP and AMP were prepared by mixing 200 mg of respective antibiotics in 1 L ultrapure water.
3. CIP was prepared by mixing 200 mg of it in 1 L of 10% acetic acid solution.

Preparation of samples for LCMS

1 mL of sample was taken whenever required into a 2 mL Eppendorf and was immediately centrifuged at 10000xg for 2-3 min. The supernatant was then collected and was immediately filtered using a 0.20 µm syringe filter to avoid particles entering LC-MS. These filtered samples were stored at -20˚C until further analysis. The samples were diluted when necessary, before analyzing them through LC-MS. Further details of the preparation of internal standards and calibration curve for LC-MS can be found in Appendix A.

3.4.4. Extraction of antibiotics from sludge

Antibiotics concentration in the sludge was determined using the similar method previously described by (Wijekoon et al., 2015). The sludge sample was centrifuged at 14000xg for 15 min, and the supernatant was discarded. After freezing the remaining sludge sample at -80˚C for at least one day, the samples were freeze-dried using a Biobase BK-FD10 series Freeze Dryer for 20-24 h. This dried sludge was then ground to a fine powder using a hand mortar and pestle. A 0.4 g of this fine powder was transferred to a tube and 4 mL of methanol was added to this tube and thoroughly vortexed using a vortex mixer for 3 min. The mixed samples were then sonicated using high-energy sonifier (Branson 450 digital sonifier) for 10 min with an amplitude setting of 20% and temperature less than 60˚C to avoid any losses of methanol due to evaporation. The sample was then centrifuged at 3300xg for 15 min, and the supernatant was collected in a fresh 15 mL tube for further analysis. A 4 mL mixture consisting of dichloromethane and methanol (1:1 V:V), was added to the sludge residue of the previous step. The process of vortexing, sonication, and centrifugation was repeated and the supernatant from this step was combined with the supernatant of the previous extraction step. Finally, this solution was filtered through a 0.20 µm syringe filter. These filtered samples were further analyzed as described in section 3.4.3 using LC-MS.

3.4.5. DNA extraction

For the DNA extraction, the sludge and effluent samples were collected once per week. Before collecting the sludge samples, the reactor was mixed well using 100 mL syringes, also the top of the reactor was purged with nitrogen to ensure good mixing. A 2 mL of homogenized sludge was then collected in a sterile 2 mL Eppendorf tube.

For the DNA extraction from permeate, as the solids concentration was less, 1 L of permeate was used. Permeate was collected in a glass bottle which was sterilized in an autoclave machine. The pipes connected to the bottle from the membrane were also sterilized before use. After the pipes were connected to the bottle, it was ensured that the atmosphere in the bottle was anaerobic by flushing the bottle with nitrogen gas for at least 3 min. Every week, around 3 L of the permeate was collected for obtaining the triplicate samples for DNA extraction. This collected permeate was then filtered through a 0.22 µm PES filter membrane of 47mm diameter. This filtration was

performed inside a laminar flow cabinet to ensure sterile conditions and avoid the contamination of filters. These filter papers were then collected in sterile Petri dishes and were cut into small pieces.

The sludge samples and effluent filters collected were then subjected to DNA extraction using FastDNA Spin kit for soil (Q-Biogene/MP Biomedicals, Solon, OH, USA) according to the manufacturer instructions mentioned in the kit. The extracted DNA was quantified by fluorometry using a Qubit 3 Fluorometer (Thermo Fisher Scientific, USA). The extracted DNA samples were then stored at -20˚C until their further use in the qPCR analysis.

3.4.6. Quantification of ARG

The DNA extracted from biomass and permeate samples as mentioned in the previous section was analyzed further using quantitative polymerase chain reaction (qPCR) for antibiotic resistant gene quantification. The standards, primers and qPCR reaction conditions used for the selected genes as shown in Table 4 can be found in Appendix A. The standards for each gene were designed using gene editor software SnapGene. qPCR reactions were carried out in 20 µL reactions with each containing, 2 µL DNA template, 18 µL of qPCR master mix. Master mix per sample consists of 0.2 µL of each forward and reverse primer (50 µM), 10 µL of SYBR green dye, 7.6 µL of qPCR grade water. Each sample was performed in technical triplicates. In each run, standards were added to generate the standard curve.

Table 4 ARGs and MGEs that were tested in this study

Group	Gene	Resistance to
ARGs	*sul1*, *sul2*	Sulfamethoxazole
	dfrA1	Trimethoprim
MGE	*intI1*	class I Integron
All bacteria	*16S rRNA*	Normalization to the concentration of bacteria

3.4.7. Quantification of bacteria

Total bacteria and ARB were quantified using the heterotrophic plate count (HPC) method. R2A agar was used for the preparation of the plate media for all the HPC plating. In total, 3 sets of plates of R2A, R2A + SMX, R2A + TMP were prepared. The agar medium (18.2 g R2A agar/L) was first autoclaved to sterilize it, then the medium was allowed to cool down at room temperature till it reached 40-50˚C. Next, the antibiotics SMX, TMP were added to the medium at concentrations of 50.4 µg/mL, 16 µg/mL respectively, and mixed well by shaking the bottles with hand. Then a 15 mL of medium was poured into each petri dish (100 X 50 mm) by pipette carefully. The plates were then dried by keeping them open in the laminar flow cabinet (Figure 5).

TMP concentration was selected based on the minimum inhibitory concentrations given in the Clinical and Laboratory Standards Institute (2015). The SMX concentration was chosen based on

the previous studies (Zarei-Baygi et al., 2020, 2019). Effluent and sludge samples were collected in 2 mL sterile Eppendorf tubes. The collected samples were then diluted using 1 X phosphate-buffered saline (PBS) solution that was sterilized before its use. A 100 µL of diluted sample was then plated in duplicates. Plates were incubated at 37˚C for 24 h before counting. The plates with 30-300 colonies were counted and their respective dilution factors were taken into account for the calculation of the total count of bacteria. This concentration was expressed as colony-forming units per milliliter of the sample (CFU/mL).

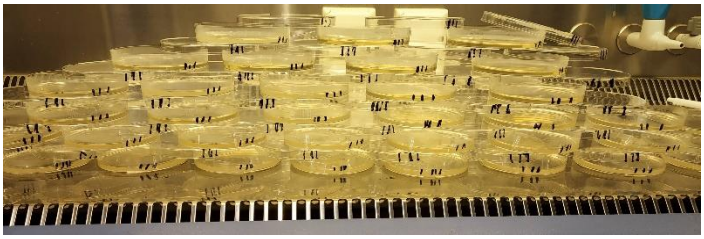

Figure 5 Agar plate drying

3.4.8. Statistical analysis methods

To determine if there is a significant statistical difference among different sets of experimental data obtained, an analysis of variance (ANOVA) test was applied using Microsoft excel. The alpha level in ANOVA analysis was set as p=0.05 in this study. If the p-value is less than the alpha level, the null hypothesis can be rejected and it can be said that there is a statistically significant difference among the data groups.

To evaluate the significant linear correlation between antibiotic concentration, ARGs and ARB, Pearson correlation was employed. Strong correlation was established if the Pearson coefficient (ρ) is > 0.7 or <-0.7. For weak correlation, $0.3 < \rho < 0.7$ or $-0.7 < \rho < -0.3$ was used.

4. Results and Discussion

4.1. Effect of antibiotics on performance of reactor

As mentioned in section 3.2, TMP and SMX were added into the reactor in two phases and kept at a final concentration of 150 µg/L. This concentration was achieved gradually in the reactor in steps of adding 10, 50, and 150 µg/L. During this whole period, the reactor was monitored continuously by collecting samples for COD, VFA, biogas, solids, and nutrients. In this section, the effect of the addition of antibiotics on the reactor operation is discussed.

4.1.1. COD removal and Biogas production

Before the addition of the antibiotics, the performance of the reactor in terms of COD removal was highly stable with 98.6±0.3% COD removal, and a low effluent COD concentration of 65±10 mg COD/L. These removal values in AnMBR are consistent with the values obtained in the literature (Luna et al., 2014; Zarei-Baygi et al., 2020). It can be seen from Figure 6 that in the first phase of addition of TMP to the reactor, initially, the COD removal was reduced to 97.4±0.4%, but after 2 weeks of operation, the COD removal was increased back to 98.1±0.6% and was almost constant in the later stages (Table 5). There was no significant variation observed in the COD removal after the 2 weeks of addition of antibiotics to the reactor (p-value = 0.82).

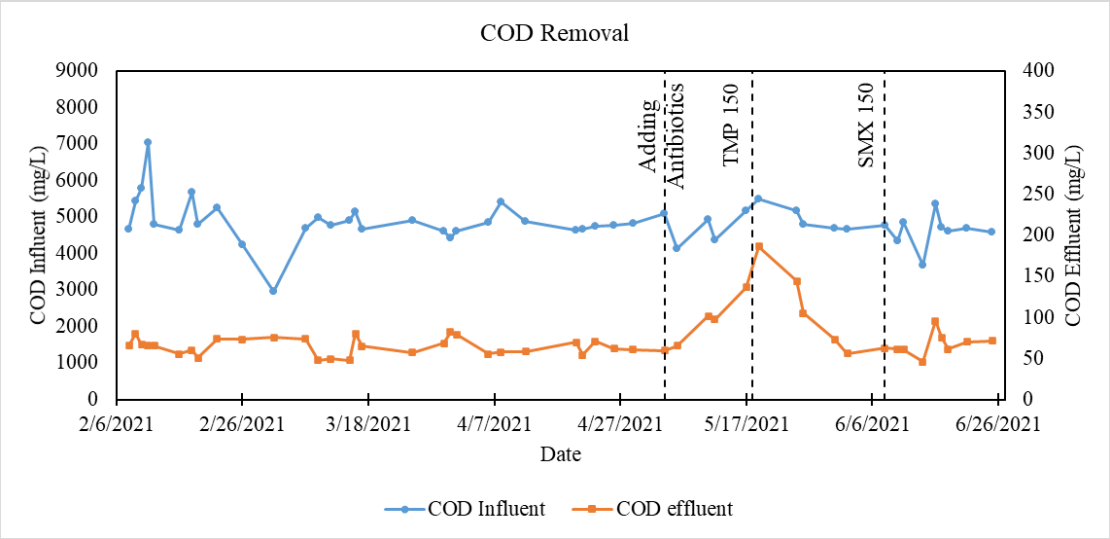

Figure 6 Removal of COD in reactor

In the initial phase of the reactor, due to the problems with recirculation, biogas production was low in the period highlighted initially in Figure 7. Later the biogas production was stable with a production of 1.79±0.53 L/day and 91±2% of methane content. The methane content in the reactor before adding limited aeration was ~82%. The added limited aeration increased the methane content by approximately 5% (Khande, 2020). Initially, the quality of biogas was high (~82%) because of the high degradability of the feed used in this study. It was observed in the previous

study conducted on the sludge collected from the same reactor by Kb (2020), that the degradability of complex compounds in the feed like cellulose and ovalbumin was around 95%. In addition to this, the solubility of CO_2 was high relatively in the water, and part of it may be chemically bound in the water (Van Lier et al., 2008). To further obtain the soluble CO_2 in the reactor, alkalinity should be measured and a mass balance should be established.

After maintaining the reactor at stable conditions for at least 1 SRT (28 days: 25th March to 3rd May), antibiotics were added to the reactor on 4th May. After the addition of antibiotics, a similar production of biogas was observed with a value of 1.74±0.73 L/day, and 88±6% of methane content (Table 5). However, the variations in biogas production and composition (Figure 7) were high in the initial stages after adding the 150 µg SMX/L to the reactor, but gradually the production increased back to normal. Nevertheless, it was observed by Cetecioglu et al. (2015), that the concentrations only above 45 mg/L of SMX were lethal on the microbial community and hence the biogas production. In several other studies, it was observed that below this concentration level, SMX has no negative effects on biogas production (Cetecioglu et al., 2016; Wang et al., 2021; Zarei-Baygi et al., 2020, 2019). In a previous study by Zarei-Baygi et al. (2020), it was concluded that after the addition of 250 µg/L of SMX to an AnMBR, there was no significant difference in the abundance of methanogens, and the microbial community of biomass was stable throughout. Even so, here, the biogas production and composition should be monitored for longer periods to check the stability of the reactor.

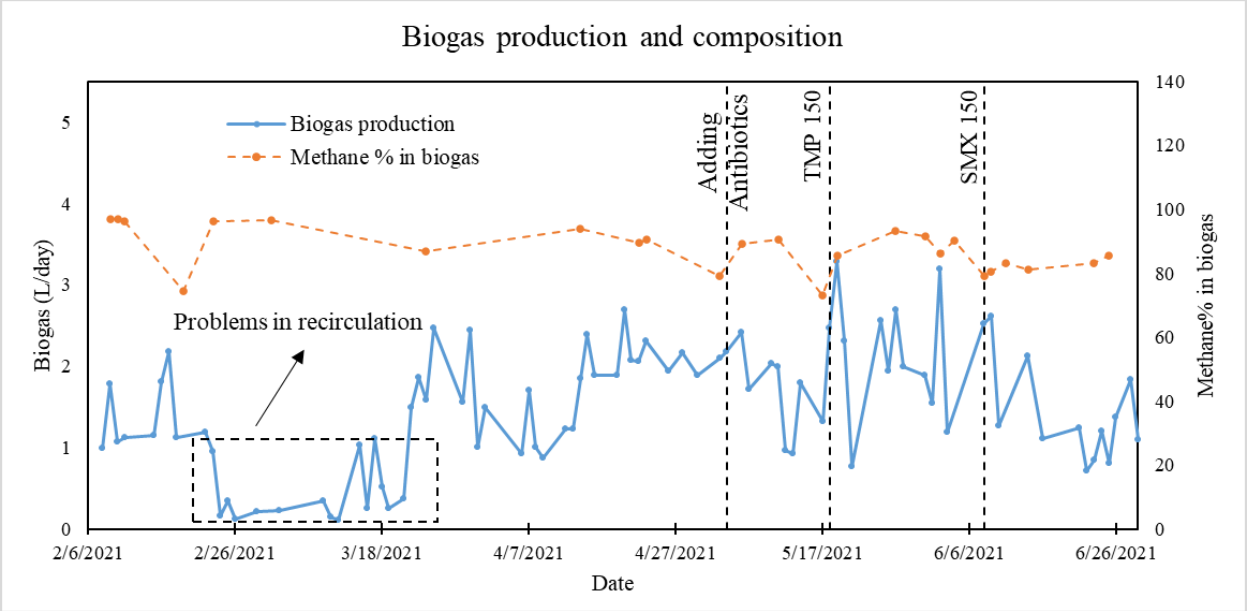

Figure 7 Biogas production and composition

Table 5 Variation in different parameters after adding antibiotics

Parameter	Before antibiotics	After antibiotics
Effluent COD mg/L	65±10	94±45
COD Removal %	98.6±0.3	98.1±0.6
Biogas production L/d	1.79±0.53	1.74±0.73
CH_4% in biogas	91.2±2.1	85.2±5.8
VFA (mg/L)	6.30±4.2	12.86±6.71

4.1.2. Nutrients removal and biomass properties

Other parameters like nutrients removal were also observed in the reactor before and after adding the antibiotics to the reactor. The difference in the removal trends can be seen in the graphs given in Appendix B. It can be observed from Table 6 that the removal of all the nutrients was not significantly different after the addition of antibiotics. The removal of phosphate in the reactor might be due to the chemical precipitation or adsorption onto the sludge (Ding et al., 2005). The precipitation of phosphate in the reactor can happen in the form of calcium phosphate or struvite. Also, the growth of biomass utilizes phosphorus as a vital nutrient (Van Lier et al., 2008).

Table 6 Removal of various nutrients

Reactor condition	Parameter	Influent mg/L	Effluent mg/L	Removal %	p-value* ANOVA
B	PO_4^{-3} - P	50±4	20±4	58±5	0.067
A		50±2	25±2	51±5	
B	SO_4^{-2}	220±32	UR*	-	-
A		280±42	UR*	-	
B	NH_4^+ - N	212±13	681±60	-221±22*	0.064
A		204±17	710±21	-251±21*	
B	TN	777±55	748±72	-	-
A		786±28	736±11	-	
B	NO_3^- - N	1.47±0.36	0.23±0.03	84±4	0.41
A		1.60±0.58	0.35±0.21	71±20	

*B indicates the reactor condition before antibiotics addition, and A indicates the condition of the reactor after antibiotics addition. *(p-value was calculated between data sets of B and A)(UR: under the range of detection)(-ve removal efficiency of ammonia indicates the accumulation of it in effluent)*

During the anaerobic treatment process, ammonification of organic nitrogen increased the concentration of ammonia in the effluent. The effluent ammonia consists of ammonia released from the degradation of ovalbumin and amide groups of urea. Also, from the nitrogen balance, it was observed that the total nitrogen present in the effluent was dominated by ammonia, and the

concentration of nitrate was very low. Also, the sulphate reducing bacteria might have reduced the sulphate present in the feed to H_2S. As the reactor was limited aerated, the part of sulphate might be converted to the elemental Sulphur. To further establish the pathway of Sulphur removal, the H_2S concentration in biogas should be measured. However, the addition of antibiotics to the reactor did not affect the removal of all the nutrients studied here.

Initially, before adding the antibiotics to the reactor, the pH was stable at 7.77±0.14 which reduced to 7.56±0.12 after the addition of antibiotics. The reduction in pH after the addition of antibiotics was due to a slight accumulation of VFAs. The major components of VFAs were acetate and propionate. The concentration of VFAs increased after the addition of the antibiotics, however, the concentration was very less to be considered lethal for microorganisms (Table 5). In addition, the parameters like total solids content, volatile solids content, and ORP with values of 7.62±1.90 g/L, 3.89±1.02 g/L, and -536±11 mV respectively, did not change after the addition of antibiotics. A detailed comparison table of biomass parameters can be seen in Appendix B. Hence, from the above study, it can be seen that the addition of antibiotics to the reactor, had a slight effect on the biogas production but had a negligible effect on the other parameters of the reactor. Nevertheless, to study the impact of antibiotics on the performance of the reactor directly, a microbial analysis should be performed.

4.2. Removal and fate of antibiotics in the reactor

The concentrations of SMX and TMP were monitored in the reactor regularly. In this section, the removal of antibiotics will be discussed.

4.2.1. Removal of TMP and SMX in reactor

As mentioned before, TMP and SMX were added to the reactor in steps of 10, 50, and 150 µg/L. The removal of TMP in each step is shown in Figure 8. The first point for measurement of effluent concentration was taken after two days (1 HRT of the reactor) of the addition of 10 µg/L. From this initial point, the removal of TMP was observed to be very high with a value of 97.3±1.3%. This value was comparable with the removal efficiency obtained in the anaerobic digestion with 90-99% removal efficiencies (Feng et al., 2017; Narumiya et al., 2013), and AnMBR with 94% TMP removal efficiency (Xiao et al., 2017). Whereas the removal of TMP in the activated sludge process was 26.4% (Li and Zhang, 2010). Hence, the limited aeration added to the reactor has no negative effects on the removal of TMP.

The removal of SMX in each step is shown in Figure 9. Similar to the TMP, the first point for measurement of effluent concentration was taken after two days of the addition of 10 µg/L SMX. From this initial point, the removal of SMX was observed to be around 86.5±2.9%. This value was comparable with the removal efficiency obtained in the anaerobic digestion with 80-98% removal efficiencies (Feng et al., 2017; Mazzurco Miritana et al., 2020; Narumiya et al., 2013). The removal of SMX particularly in AnMBR ranged from 68% to 90% (Wei et al., 2019; Xiao et al., 2017;

Zarei-Baygi et al., 2020). Whereas the removal of SMX in the activated sludge process was 39.1% (Li and Zhang, 2010). Hence, the limited aeration did not reduce the SMX removal.

Due to the presence of the substituted functional groups of electro withdrawing natured sulphonyl group, and substituted pyrimidine group on SMX and TMP respectively, the degradation occurs well in anaerobic conditions for these antibiotics (Alvarino et al., 2018, 2016). In this study, although the reactor was added with limited aeration of 14.7 mL air/Lrec/d, the removal in SMX, TMP was found to be high and according to literature, it might be unaffected by limited aeration. However, as the added aeration in this study, helps the reactor to improve in other parameters like COD removal and biogas production (Khande, 2020), it is advantageous to see that there was no negative effect of limited aeration on the removal of selected antibiotics.

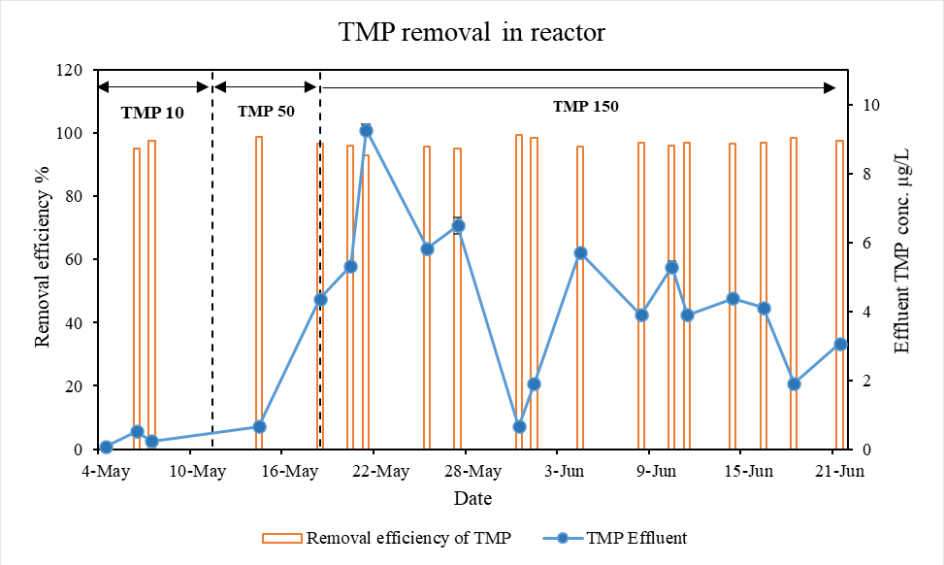

Figure 8 Removal of TMP in reactor

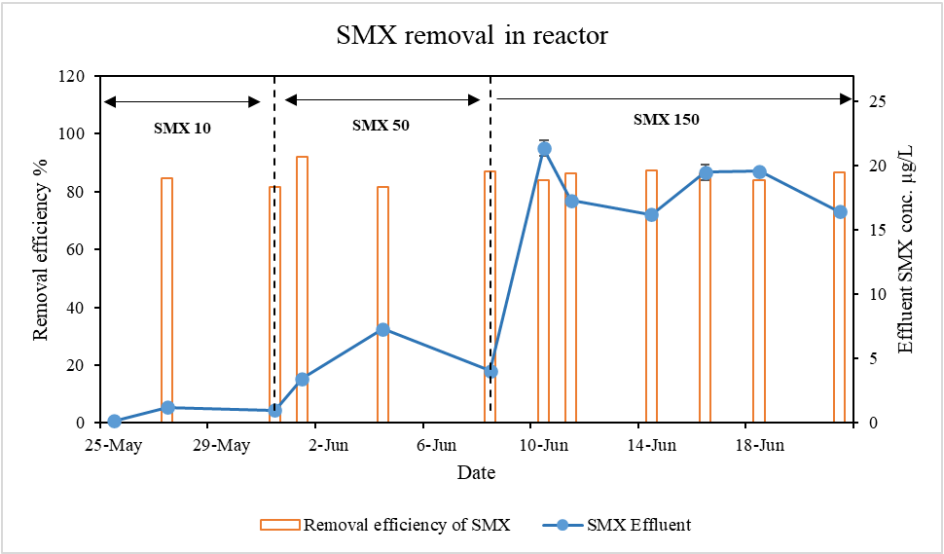

Figure 9 Removal of SMX in reactor

4.2.2. Fate of TMP and SMX in reactor

Besides monitoring the antibiotics (TMP and SMX) concentration in feed and effluent, the amount of antibiotic adsorbed onto biomass was also measured. To determine the removal mechanism of antibiotics inside the reactor, a mass balance was carried out from the concentration of antibiotics obtained from feed, effluent, biomass inside the reactor, and the wasted sludge from the reactor. The volatilization of these antibiotics can be eliminated due to their low Henry's constant values. As the concentrations of antibiotics from the feed bucket and feed sampling point located just above the reactor were similar, the removal due to accumulation or adsorption in the feed pipe can be considered negligible. In addition, as the mixing of the reactor was good, it can be assumed that the removal due to sorption on the glass reactor walls was zero. Thus, the only possible removal mechanisms for antibiotics would be adsorption, biodegradation, discharge through effluent, or sludge wasted. Hence, the following mass balance was established in the reactor.

$$AB_{inf} = AB_{sorption} + AB_{WS} + AB_{Eff} + \text{Biodegradation}$$

Where AB_{inf} and AB_{Eff} are loads of antibiotics in the influent and effluent of the reactor, AB_{WS} is the amount of antibiotic wasted through the sludge removed in the reactor daily, and $AB_{sorption}$ is the amount of antibiotic adsorbed onto the sludge in the reactor. From all the remaining terms of the equation, the amount of antibiotic biodegraded can be known, which is shown as Biodegradation in the equation. Further details of the calculation can be found in Appendix C.

It can be observed from Figure 10 that in the initial stages of TMP 150 µg/L feeding, removal due to adsorption was 5%, which gradually reduced to 2% at the end of the study. From the starting day of TMP 150 µg/L, the biodegradation or biological transformation was the main removal mode of TMP. Since the values in the first 2 weeks where TMP 10 and 50 µg/L were added to the reactor were unknown, it can't be established whether initially, the removal mode was due to adsorption, biodegradation, or discharge through the permeate. However, from Alvarino et al. (2018) and Feng et al. (2017), it was observed that the TMP would degrade rapidly in the low ORP range, due to the presence of a substituted pyrimidine functional group, that can be readily biotransformed. After the addition of SMX 150 µg/L, the TMP adsorbed was increased back to 5% (15-June), this might be due to the disturbance in microbial diversity due to SMX addition. However, the adsorbed TMP reduced back to 2% by 18-June. At the end of the study period, the discharge of TMP through permeate was reduced to 0.5% from a maximum of 5% (Figure 10).

The removal pathways for SMX can be seen in Figure 11. For SMX, the biodegradation process was the main removal mode since the initial addition of SMX to the reactor. The negative charge of SMX makes the adsorption process onto biomass almost impossible. Methylation, cleavage, and hydroxylation of the isoxazole ring of SMX are some of the possible routes of co-metabolism of SMX (Jia et al., 2017). The loss of amino groups from the aniline ring of SMX might also be a possible degradation mechanism of SMX in anaerobic conditions (Carneiro et al., 2020).

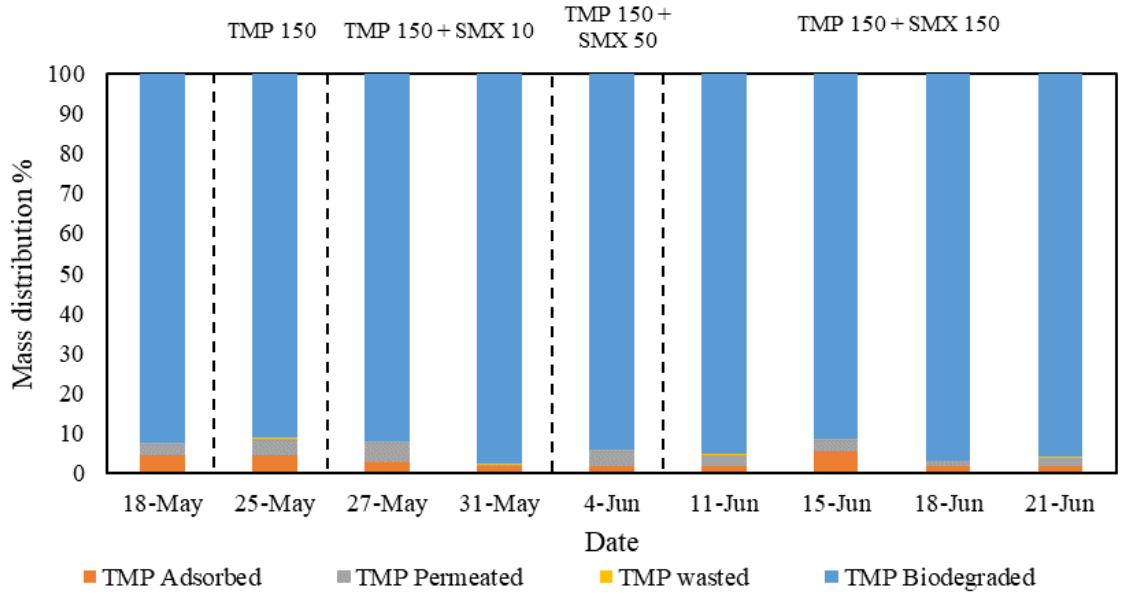

Figure 10 Mass balance of TMP in reactor

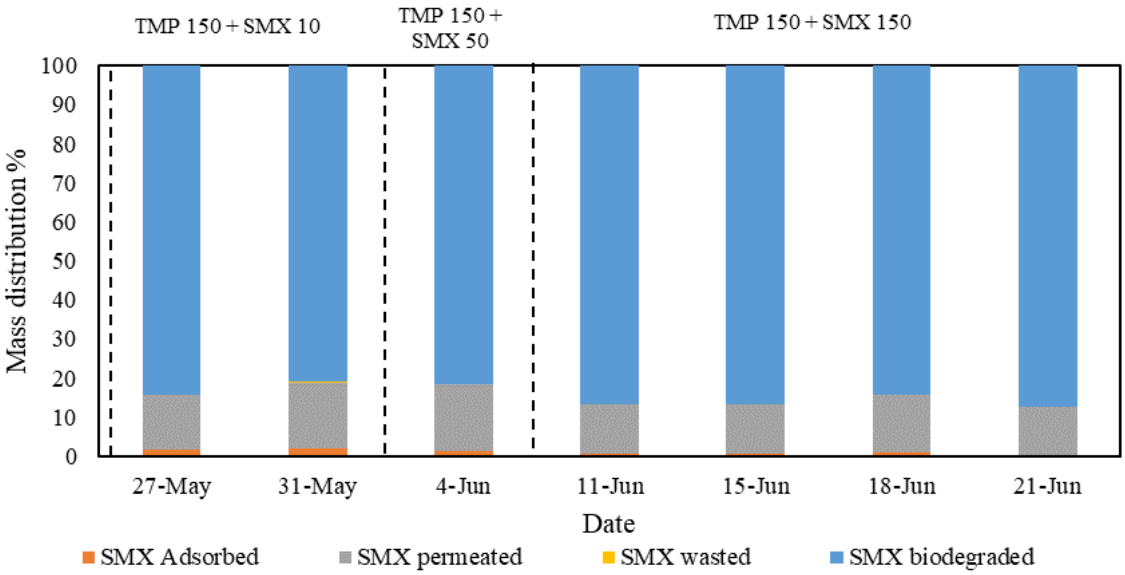

Figure 11 Mass balance of SMX in reactor

4.3. Adsorption of antibiotics onto biomass

To analyze the removal mechanisms found in earlier sections, further, adsorption and biodegradation were studied through batch tests. To obtain the adsorption isotherms and analyze the effect of initial concentration on their removal efficiencies, adsorption tests were performed at two different temperatures of 10°C and 37° and three concentrations of 10, 50, and 150 µg/L.

4.3.1. Tests at 10°C

The graphs of the removal of TMP at 10°C are shown in Appendix C. At all the concentrations verified, for TMP, it was observed that most of the adsorption took place within the first 5 mins. This is because of the more adsorption sites present on the biomass initially. After 5 mins, as TMP occupied the adsorption sites of biomass, with time the adsorption rate reduced, and the equilibrium was reached. In addition to the available adsorption sites, the property of adsorbate is important for adsorption. TMP with its high Log K_{ow} value and positively charged nature, adsorbed onto negatively charged biomass quickly (Jia et al., 1996). The removal efficiencies at all concentration levels was around 82% as shown in Table 7. The p-value obtained from ANOVA statistical analysis had a value of 0.19 which implies a negligible effect of varying concentration on their removal efficiencies.

On the other hand, removal of SMX via adsorption was very less with a removal efficiency of 7% on average (Appendix C). This less removal via adsorption was similar to as observed in previous studies (Khande, 2020; Narumiya et al., 2013). The reason for the less removal is because of the negative charge of the SMX which makes it difficult to adsorb onto negatively charged biomass. Also, the Log K_{ow} value of SMX is less which shows the lower affinity of SMX to biomass. Similar to the TMP, the p-value obtained from ANOVA statistical analysis had a value of 0.47 showing the negligible effect of varying concentration on their removal efficiencies.

Table 7 Removal efficiencies of SMX and TMP at different concentrations at 10°C

Conc. ug/L	Removal TMP %	Removal SMX %
10	80.8±7.5	6.7±0.6
50	85.4±6.2	11.1±5.2
150	82.4±7.4	10.6±3.6
p-value (ANOVA)	0.19	0.47

The adsorption isotherms for SMX and TMP were analyzed using linear and Freundlich isotherms. The equations used are explained here.

Linear $q_e = K_d * C_e$

Freundlich $q_e = K_f * C_e^n$

K_d is the linear sorption coefficient, q_e is the concentration of antibiotic sorbed onto sludge in µg/g and C_e is the concentration of antibiotics at equilibrium in the water phase (µg/L). K_f is the Freundlich coefficient and n is the Freundlich exponent. The Freundlich isotherm describes non-uniform distribution with different affinities of adsorption on a heterogeneous surface (Sun and Selim, 2020). The linear isotherm is the simple case where the affinity of the antibiotic remains constant over the concentration level.

It was found that the coefficients of correlation were larger than 0.98 for both antibiotics with two models (Table 8), which shows that both isotherm models could be used to describe the sorption of SMX and TMP effectively. However, as the points used for constructing the isotherms were few (three), it is important to check the applicability of the isotherms also in the larger population. As the p-value for linear isotherms was less than 0.05 (usual significance level), the linear isotherms best fit the adsorption isotherms for both SMX and TMP (Table 8).

The K_d value obtained for SMX was 0.029 L/g which implies that the adsorption of SMX was less. Whereas for TMP, the value was 1.234 L/g, which is higher than the values obtained on the primary and secondary sludge with values of 0.39 and 0.42 L/g respectively (Hörsing et al., 2011).

Table 8 Parameters of adsorption isotherms

Linear isotherm				
Antibiotic	**K_d (L/g)**	**n**	**R^2**	**p-value**
SMX	0.029	1	0.998	0.001
TMP	1.234	1	0.996	0.002
Freundlich isotherm				
Antibiotic	**K_f**	**n**	**R^2**	**p-value**
SMX	0.011	1.212	0.980	0.090
TMP	1.148	1.046	0.988	0.069

4.3.2. Tests at 37°C

As the adsorption process is temperature dependent, to understand the removal of antibiotics through adsorption at the reactor operating conditions, the adsorption tests were also performed at 37°C. The adsorption trends for both SMX and TMP are similar to the adsorption at 10°C (Appendix C). Nonetheless, the removal of SMX reduced from 7% at 10°C to almost negligible at 37°C. Removal of TMP observed was in the similar range around 85% as observed at 10°C for lower concentrations (Table 9), however, the biomass used for the tests at 37°C has high solids

content compared to that used for tests at 10°C. Hence, the difference can be seen in the linear isotherm coefficient of TMP (Table 10), which is lower at 37°C (0.513 L/g) compared to 10°C (1.234 L/g). With an increase in temperature, the adsorption potential of TMP was reduced. The movement of antibiotics from solid to bulk phase might have increased with the rise in temperature (Bekçi et al., 2006). Hence to predict the exact mechanisms in the reactor, the tests should be conducted at 37°C.

Table 9 Removal efficiencies of SMX and TMP at different concentrations at 37°C

Conc. ug/L	Removal TMP %	Removal SMX %
10	90.1±5.1	5.9±0.2
50	84.7±2.2	1.8±8.2
150	66.6±5.9	2.6 ±0.5
p-value (ANOVA)	0.009	0.674

Table 10 Linear isotherm parameters of TMP at 37°C

Linear isotherm Parameter	Value
K_d (L/g)	0.513
R^2	0.926
p-value	0.037

4.4. Degradation of antibiotics

Two types of degradation tests were performed to enquire about the effect of removal efficiencies on the feeding patterns. In the one-time feeding tests, the calculated amount of acetate was given on the first day of the experiment, while in the intermittent feeding tests, the calculated acetate was supplied five times in the first five days of the experiment. The intermittent feeding test was chosen to simulate the reactor conditions to the maximum extent possible in the batch tests.

4.4.1. One-time feeding

The one-time feeding test was conducted for ten days until the given acetate was totally consumed. The removal of SMX and TMP with an initial concentration of 150 µg/L is given in Figure 12. The removal of both SMX and TMP was more than 98%. To understand the removal kinetics, a first-order kinetic model was applied to fit the degradation data.

First − order kinetics: $C_t = C_0 * e^{-k.t}$

C_0 is the initial concentration of the antibiotic, C_t is the concentration of antibiotic at time t, and k is the first-order rate constant. With this equation, the half-life is calculated as $t_{1/2}$ = ln 2/k.

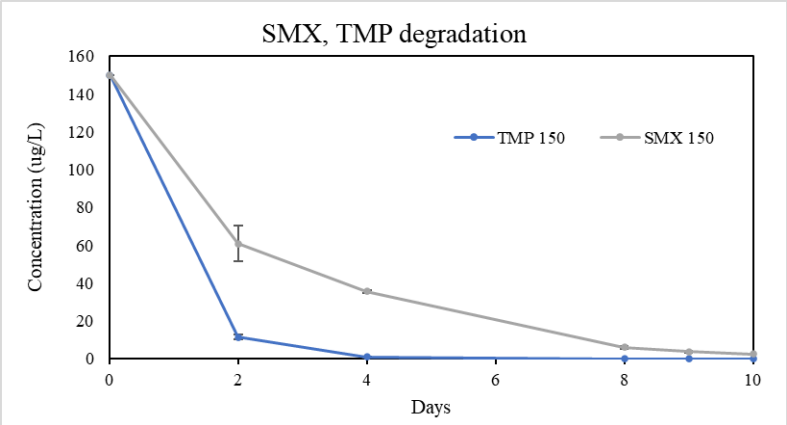

Figure 12 Removal of SMX, TMP through degradation tests with one-time feeding

Table 11 Parameters of the first-order kinetics model

	k (d^{-1})	**$t_{1/2}$ (days)**	**R^2**
SMX 150	0.4047	1.71	0.997
TMP 150*	0.8271	0.84	0.881

**For TMP the values might be affected due to the initial adsorption process*

It can be observed from Table 11 that the coefficient of correlation was high for SMX with a value of 0.99, as the removal of SMX due to adsorption was less, the main mode of removal is considered to be biodegradation, hence the first-order kinetic fit is well established. It can be seen that the half-life of SMX is 1.71 days, which is higher compared to TMP (0.84 days). The rate constant of SMX is higher compared to the values obtained using the activated sludge, which has a rate constant value of 0.264 d^{-1} and a half-life of 2.67 days (Li and Zhang, 2010).

4.4.2. Intermittent feeding

Intermittent feeding batch tests were established to simulate the reactor condition to a maximum extent as mentioned in section 3.3.1. The removal pattern of SMX and TMP were similar to that of the onetime feeding batch tests (Appendix C). In this set of tests, to know the fate of antibiotics, in addition to the antibiotic in the liquid phase, the amount adsorbed onto the solids phase (sludge) was also measured to establish the following mass balance at the end of the test.

$$AB_{input} = AB_{adsorbed\ on\ sludge} + AB_{liquid\ phase} + Biodegradation$$

Where, AB_{input} is the amount of biomass inputted in the bottle, $AB_{adsorbed\ on\ sludge}$ is the amount of antibiotic adsorbed onto the solid phase, $AB_{liquid\ phase}$ is the amount of antibiotic present in the

liquid phase of the bottle, and Biodegradation is the amount of the given antibiotic biodegraded in the period. The results of this mass balance can be seen in Table 12.

Table 12 SMX and TMP removal via different mechanisms in degradation batch test 2

Removal mode of AB	SMX %	TMP %
Adsorption	0.1	0.1
AB Present in liquid phase	0.1	0.1
Biodegradation	99.8	99.8

It can be observed from Table 12 that the main removal mode of both antibiotics was biodegradation/biotransformation. From this and section 4.3 the predicted mechanism of removal for TMP via the degradation set of batch tests can be established as first adsorption, followed by biodegradation. For SMX, the bulk removal can be directly due to co-metabolism or biodegradation. Total removal of SMX and TMP were high with >99% removal and <1% being ended up in the liquid phase.

4.5. Antibiotic resistance

In this study, SMX resistant genes *sul1* and *sul2*, TMP resistant gene *dfrA1* were analyzed. In addition, class 1 integrons of *intI1*, and 16s rRNA were also quantified. ARGs of biomass and effluent were normalized against the volume as gene copies/mL.

4.5.1. Development of antibiotic resistance in biomass

The ARGs and *intI1* present in the biomass at different stages of antibiotic addition is shown in Figure 13 (i). Two spikes can be seen in the concentration of ARGs in biomass, which occurred after the addition of 150 µg/L concentration of TMP and SMX respectively. With the addition of TMP 150 µg/L, the abundance of all the ARGs *dfrA1, sul1, and sul2* increased in the biomass. On the other hand, with the addition of the highest concentration of SMX 150 µg/L, only *sul2* was increased significantly.

Among the SMX resistant genes, *sul2* was found to be more abundant than *sul1* in biomass. The concentration of the *sul2* in the reactor before the addition of antibiotics was 2E+09 copies/mL which increased to 7E+09 copies/mL after the addition of SMX 150 µg/L. This trend of increase in the abundance of *sul2* with the antibiotic concentration was in line with the previous studies (Blahna et al., 2006; Zarei-Baygi et al., 2019). The abundance of *sul1* was increased from 5E+08 to 9E+08 copies/mL in the biomass after the addition of TMP 150 µg/L, however, it reduced to 4E+08 copies/mL after the addition of SMX to the reactor. Similarly, the abundance of *dfrA1* was increased from 8E+07 to 2E+08 copies/ mL after adding the TMP 150 µg/L and later reduced to 5E+07 copies/ mL after the addition of SMX. This might suggest that the genes *sul1* and *dfrA1* are not responsible for the development of resistance to SMX. In previous studies, it was observed that the abundance of *sul1* was highly correlated with SMX concentration (Hsu et al., 2014; Zarei-

Baygi et al., 2019), however, in this study the reduction in *sul1* was observed with SMX concentration and should be studied further. A slight increase was also seen in the abundance of class 1 integrons after the addition of TMP 150 µg/L. The class 1 integrons are present on the mobile genetic elements (MGEs) like plasmids. This MGEs abundance helps with the horizontal gene transfer as mentioned in section 2.3. Hence, the increase in *intI1* abundance after the addition of a high concentration of antibiotics in this study can indicate an increase in MGEs and further the HGT among the microorganisms in biomass. The increase of ARGs in the selective pressure of high antibiotic concentration applied was also reported by Zarei-Baygi et al. (2020, 2019).

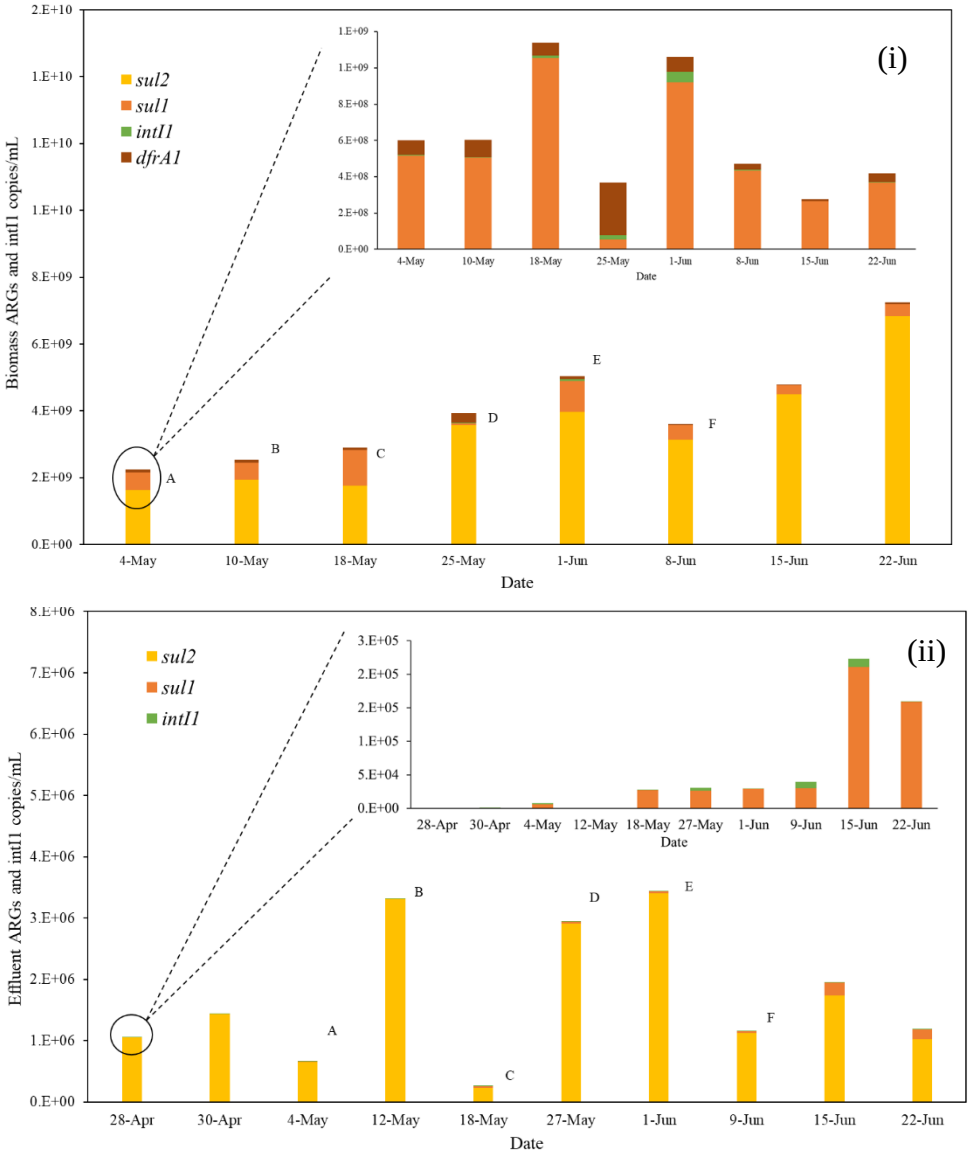

Figure 13 Abundance of ARGs in biomass (i) and effluent (ii) of the AnMBR
Points A to F indicate stages of the addition of TMP, SMX to reactor, A: TMP 10 µg/L, B: TMP 50 µg/L, C: TMP 150 µg/L, D: SMX 10 µg/L + TMP 150 µg/L, E: SMX 50 µg/L + TMP 150 µg/L, F: SMX 150 µg/L + TMP 150 µg/L
*The samples for the analysis were taken just before the addition of increased concentration

4.5.2. Development of antibiotic resistance in effluent

The presence of membrane reduced the abundance of ARGs of *sul1, sul2, and dfrA1* respectively by 3.2 log, 3.6 log, and 7.3 log units. In addition, the class 1 integrons and 16s rRNA were removed by 3 log and 3.2 log units respectively. This removal was observed to be in a similar range as mentioned in the previous study (Kappell et al., 2018a).

The relative abundance of ARGs in biomass and effluent were found to be similar, however, the trends were different. In effluent, *sul2* was the most abundant gene whereas *dfrA1* was not found. From Figure 13 (ii), it can be seen that after the addition of antibiotics to the reactor initially, a sudden rise in ARGs was found (12-May), predominantly due to *intI1* abundance along with *sul2*. The *sul2* and *intI1* are found to be co-located on conjugative plasmids generally. The gene cassettes with *sul2* and *intI1* are found abundantly in the wastewater (Zheng et al., 2017). In addition, this can also be seen from the correlation coefficient between the abundance of *sul2* and *intI1* which was 0.73 (Appendix C). This might indicate that the integrons are responsible for the presence and distribution of ARG *sul2*. The increase of the antibiotic concentration increased the horizontal gene transfer in the biomass and effluent as explained in the earlier section. This HGT might have increased the extracellular plasmid DNA, which hence intensified the harboring of plasmid-based resistance within the microorganisms (Chaturvedi et al., 2021). Unlike biomass, in the effluent, the abundance of gene sul1 was found to be increasing constantly. This might also indicate the growth of the ARGs on the post membrane part of the reactor.

The antibiotic resistant bacteria developed in the effluent is shown in Figure 14. The TMP-resistant bacteria (RB) emerged in the effluent after the addition of 10 µg/L of SMX to the feed. The presence of the TMP resistant bacteria (TMP RB) was strongly correlated to the presence of gene *sul1*. Similarly, the SMX resistant bacteria (SMX RB) appeared in the effluent after the addition of 50 µg/L of SMX and had a high correlation with the presence of gene *sul1*. TMP RB, SMX RB, and *sul1* showed a high correlation also with the SMX concentration. The correlation of the presence of TMP RB and SMX RB was high with a value of 0.99 (Appendix C), which indicates that most of the bacteria developed multidrug resistance. Although the presence of TMP RB was higher in effluent compared to the SMX RB, the presence of TMP resistance genes *dfrA1* was negligible in the effluent. However, the high correlation of TMP RB with *sul1* may indicate that *sul1* developed multidrug resistance. To support this, more TMP resistance genes like *dfrA12, dfrA13, dfrA17,* etc. should be quantified. Almost all the bacteria present in the effluent gained resistance to either TMP or SMX or both the antibiotics after adding the concentration of 150 µg/L SMX. The development of this resistant bacteria might be mostly due to the presence of ARGs and class 1 integrons (*intI1*). However, as the reactor did not reach a stable condition yet, the development of ARB and ARGs should be monitored further.

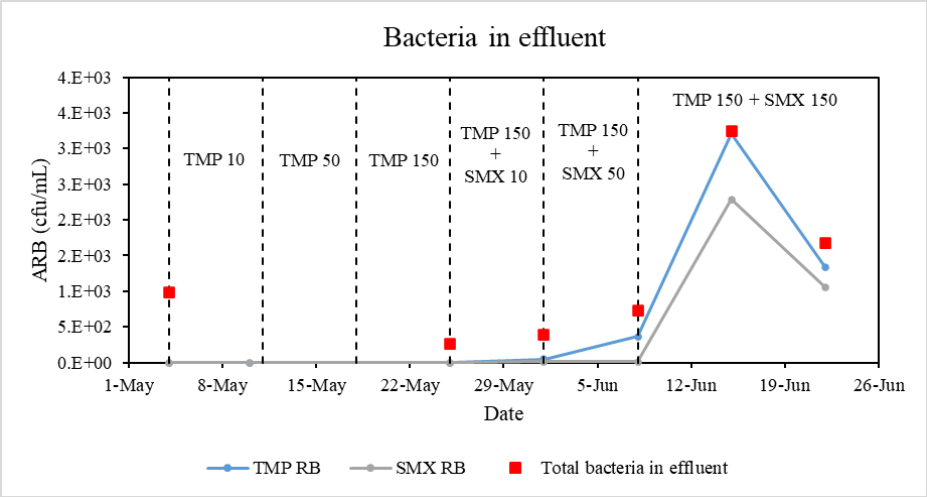

Figure 14 Antibiotic resistant bacteria in effluent

4.6. Removal of CIP and AMP through batch tests

To study the effect of limited aeration on the removal of antibiotics of CIP and AMP, batch experiments were established similarly as mentioned in section 3.3.1, with onetime feeding and intermittent feeding.

4.6.1. One time feeding

Three different concentrations of 10, 50, and 150 µg/L were used in this study. The onetime feeding batch tests were conducted for ten days, till the acetate was completely consumed. Figure 15 shows the removal of CIP and AMP at the end of ten days of the experiment. It can be seen that the removal efficiencies of CIP and AMP at all initial concentrations were higher than 80%. The p-values for the data set of CIP and AMP at varying concentrations were 0.14 and 0.15 respectively (>0.05). Hence, it can be concluded that the initial concentration of CIP and AMP within the range considered in the current study, does not affect their removal efficiencies. According to Thi Mai (2018), the removal efficiencies of CIP will be affected highly with the initial concentration of CIP > 1.5 mg/L (Thi Mai, 2018).

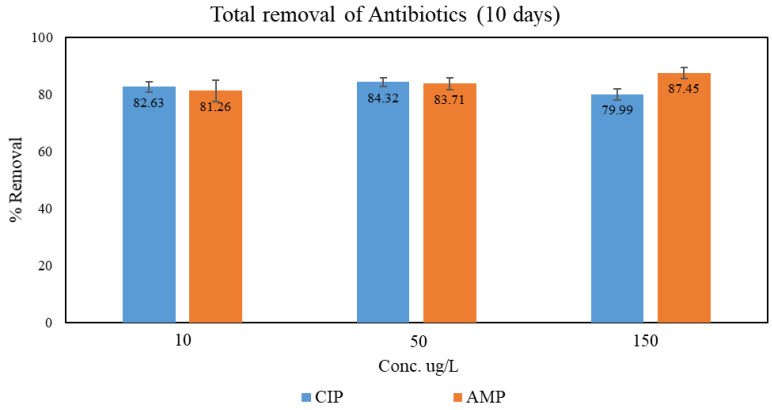

Figure 15 Removal of CIP and AMP at different concentrations

4.6.2. Intermittent feeding

The removal patterns of CIP and AMP in the intermittent batch tests were similar to that of the onetime feeding batch tests (Appendix D). For AMP, the removal efficiencies in both intermittent feeding and onetime feeding were similar with a value of ~88% (Figure 16). However, for CIP, the removal efficiency in the intermittent feeding batch tests reduced to 60% in comparison with the onetime feeding batch tests where the removal was 80%. It might be due to manual error in the measurement of CIP, or there might be a negative effect of feeding patterns on the microbes related to the degradation of CIP. However, to confirm this further, microbial community analysis must be performed to verify the change in communities in onetime and intermittent feeding batch tests.

To know the fate of CIP and AMP, the mass balance was established similarly as mentioned in section 4.4.2, after 28 days of the experiment. The removal efficiency of AMP was high (96%), which was due to biodegradation/biotransformation. The removal of AMP in anaerobic reactors was found to be around 24-30% (Huang et al., 2018b), whereas according to Shen et al. (2010) the removal of AMP in the airlift biofilm reactor was observed to be high with 90-98%. Hence, it can be said that in this study, the added limited aeration in the batch tests might have improved the removal of Ampicillin by enhancing its biodegradation. On the other hand, for CIP the effects of limited aeration seem to be negligible as the removal was similar to that found in anaerobic digestors (50-76%) (Thi Mai, 2018). However, the removal via onetime feeding test was found to be significantly high. To further study and verify the effect of limited aeration on removal of CIP and AMP well, the removal should be investigated with varying aeration concentrations.

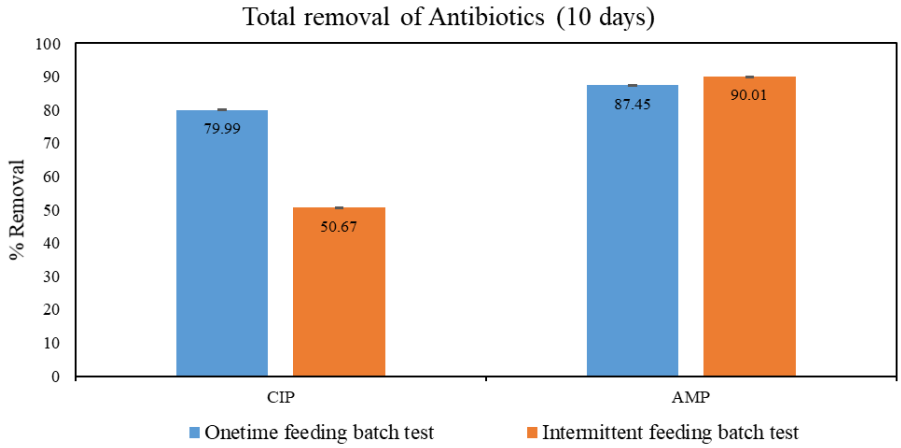

Figure 16 Removal efficiencies of CIP and AMP in two batch tests

Table 13 Removal of CIP, AMP via different mechanisms in degradation batch test 2

Removal mode of AB	CIP %	AMP %
Adsorption	2.2	0.1
AB Present in liquid phase	39.9	4.3
Biodegradation	57.9	95.6

For CIP and AMP, a preliminary adsorption batch test was performed with 150 µg/L concentration of each antibiotic at 10˚C as mentioned in section 3.3.2. The removal observed was 40% and 90% respectively for CIP and AMP which is in accordance with the log K_{OW} value for AMP (Z. Chen et al., 2020). For CIP, no adsorption was expected as it has a very low log K_{OW} value, however, due to its positive charge, the adsorption might happen due to the electrostatic interactions between CIP and biomass (Thi Mai, 2018). Also, via degradation batch tests, it was observed that the antibiotics CIP and AMP, did not affect the biogas production (details in Appendix D). Hence, these two antibiotics can be added to the AnMBR directly in the future, to study their removal.

4.7. Extended discussion

The results obtained from the adsorption batch tests indicated removal of 85% for TMP, which implies that in the reactor, TMP is getting adsorbed onto the biomass initially, followed by biodegradation. This removal mechanism is very efficient as the initial adsorption process increases the substrate TMP concentration around the adsorption sites present locally in the reactor, which makes the biodegradation more thermodynamically favorable (Xiao et al., 2017). In addition, the adsorbed TMP on sludge stays in the reactor for a longer time than the usual HRT of the reactor, which causes an increase in the available amount of substrate TMP that can be degraded.

Whereas for SMX, the adsorption was almost negligible in batch tests, implying that in the reactor, the only pathway for its removal is direct uptake by microorganisms (biodegradation). Hence the removal efficiencies are comparatively low for SMX in reactor than that of the batch tests with the higher residence time. This removal of SMX in the reactor can be increased by adding adsorbents like activated carbon to the reactor, which can increase the adsorption of SMX and hence the biodegradation later. From Figure 17, it can be observed that the removal of SMX increased from 60% to 98% from day 2 to day 10 of the experimental period. Hence, the removal of SMX in the reactor might also be increased by increasing the HRT of the reactor. On the other hand, the removal of TMP was not affected by the time of the experiment, hence the removal of TMP might not vary much with a change in HRT. However, from Table 14, it can be seen that the removal of both TMP and SMX through the biotransformation pathway increased from 95% and 85% respectively to 99% with a 28 day time period in the intermittent feeding batch test. Hence, improving the HRT can improve not only the total removal but also the removal through biodegradation.

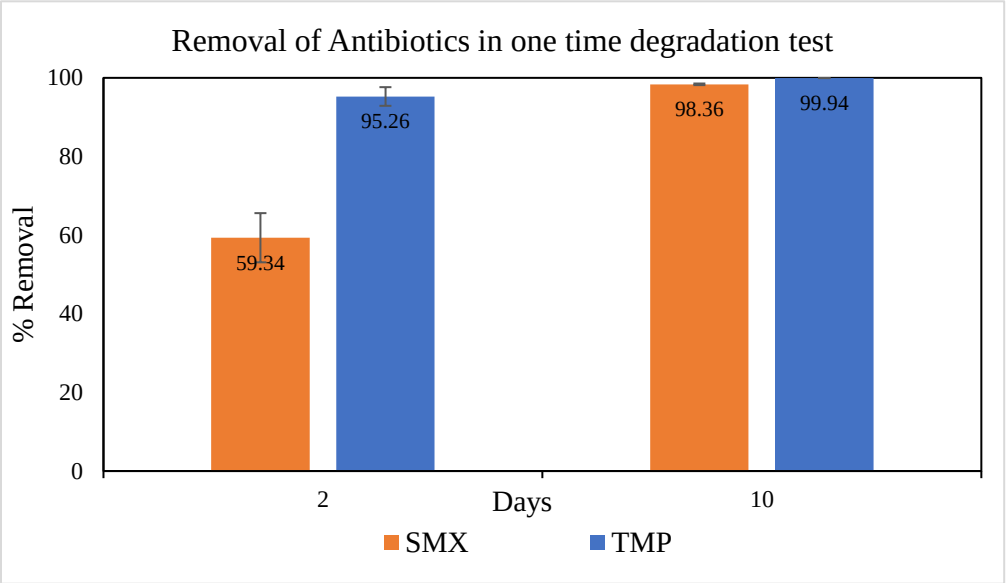

Figure 17 Removal of SMX and TMP in 2 and 10 days of biodegradation test with onetime feeding

Table 14 Comparison between results of IF batch test and Reactor

Removal mode of AB	**TMP %**		**SMX %**	
	Reactor	IF batch test	Reactor	IF batch test
Adsorption	3.0	0.1	1.1	0.1
Biodegradation	94.1	99.8	84.5	99.8
Effluent	2.8	0.1	14.3	0.1

As the method used for extraction of the antibiotics from the adsorbed sludge was implemented the first time, the method was validated by performing the antibiotic extraction at the end of an adsorption experiment for both SMX and TMP 150 µg/L. The results obtained showed that the accuracy of the method was ~80%. For obtaining more accurate results, solid-phase extraction can be used as described in Wijekoon et al. (2015).

The CIP and AMP can be further studied similarly to SMX and TMP by adding them to the AnMBR. Based on the results of SMX and TMP obtained from reactor and degradation batch tests, it can be expected that the highly adsorbing AMP might have high removal efficiencies similar to the range of TMP whereas CIP might have lower removal efficiency. It was observed that the addition of CIP (>0.5 mg/L) to the AnMBR can lead to changes in the microbial community by the accumulation of propionate in the reactor due to the reduction of *Syntrophobacter* (Thi Mai, 2018). On the other hand, as the antibiotic AMP was not studied extensively, its effects on the AnMBR performance are unknown.

It was observed from this study that the addition of antibiotics to the reactor had no negative effects in terms of the performance of the reactor. The COD removal and biogas production was reduced by ~2% initially after the addition of antibiotics, but after a few weeks, the values increased back to their original state. Hence the first hypothesis "A*ddition of antibiotics to reactor reduces the performance of AnMBR by accumulating the VFAs and effecting the COD removal efficiency, biogas production, and nutrients by more than 10%*" can be refuted. However, the addition of antibiotics in higher concentrations might still affect the performance of the reactor. Hence, to establish the safe range of antibiotics concentration addition to the reactor, further studies should be performed.

Further, the removal of AMP was more than 80% in this study. However, from the literature, the removal in anaerobic conditions was expected to be around 40%. Hence the literature points to a possibility that the added limited aeration to the anaerobic conditions, might have improved the removal of antibiotics. On the other hand, for CIP, the removal was found to be almost similar to the observed range (50-70%) in the literature. Hence, the third hypothesis "*The removal of antibiotics CIP and AMP can be improved by the addition of limited aeration compared to the anaerobic conditions by at least 20%*", holds partially. To further validate the third hypothesis, a few more tests including the varying aeration concentrations, and other parameters (HRT, F/M ratio, etc.) that might impact the removal of these antibiotics, should be studied.

This research showed that the limited aeration assisted AnMBR, can significantly reduce the presence of antibiotics (~85%), ARGs, and ARB to a greater extent (~4 log units) compared to the conventional activated sludge process. Hence the second hypothesis holds. However, almost all the bacteria present in the effluent became resistant to the antibiotics. Even so, as the concentration of ARB and ARGs were still found to be less in the effluent, using an additional post-treatment step can help in improving the quality of effluent further, and can be considered in reusing the water for potable purposes.

5. Conclusions

The analysis of removal of antibiotics and antibiotic resistance was performed in this research and the main conclusions are presented in this section.

- Overall COD and nutrients removal remained unaffected with the addition of antibiotics. Biogas production and composition was affected slightly after the addition of SMX 150 µg/L, but eventually, it reached back to the initial value
- Total removal of SMX and TMP in the reactor was 86% and 97% respectively. Biodegradation was the main removal pathway for both SMX and TMP. Adsorption of SMX and TMP onto sludge contributed to only 1% and 2% respectively for total removal. Very little amount (~3%) of TMP was getting discharged through effluent, but for SMX around 14% of it was discharged
- Removal of TMP observed through adsorption via batch tests was high irrespective of the initial concentration chosen in this study. Based on R^2 and p-values, linear adsorption isotherm gave a better fit with K_d value of 1.23 L/g at 10˚C and 0.51 L/g at 37˚C, indicating a reduction in adsorption potential with an increase in temperature. Adsorption of SMX was negligible (<10%) at all concentrations and temperatures verified
- Removal of SMX and TMP was high in degradation batch tests (>98%). The SMX followed first-order degradation kinetics with a rate constant of 0.41 d^{-1} and a half-life of 1.71 days. The removal of SMX and TMP through degradation batch tests was independent of the feeding pattern used (onetime/intermittent). At the end of intermittent feeding batch tests, the main removal mechanism for both antibiotics was observed to be biodegradation
- The ARGs and ARB increased with the addition of antibiotics. The presence of membrane reduced the abundance of ARGs of *sul1, sul2, and dfrA1* respectively by 3.2 log, 3.6 log, and 7.3 log units, and class 1 integrons by 3 log units. After the addition of SMX to the reactor, almost all the bacteria present in the effluent gained resistance either to TMP or SMX or both. Among the studied ARGs, the main genes responsible for the resistance development were *sul1* and *sul2*. The correlation between the presence of *sul1* and TMP RB, *sul1* and SMX RB was high (~0.9), indicating that the gene *sul1* might have been involved in multidrug resistance
- Removal of CIP and AMP was ~82% and ~84% respectively via one-time feeding degradation batch tests. The initial concentration of these antibiotics has negligible effect on their removal. Feeding pattern has no effect on the removal of AMP, whereas the removal of CIP reduced to 60% in the IF batch tests. Biodegradation was the main removal mechanism for CIP and AMP. The effect of the CIP and AMP on the biomass was negligible with similar production of biogas observed through batch tests and can be added to the reactor for their analysis in future
- The removal of AMP was significantly high in the limited aeration assisted tests, while CIP, had no effect. However, to confirm the effect of limited aeration, further tests should be performed

6. Recommendations

The following recommendations were made based on the results of the present study, to fill in the research gaps further and answer several questions regarding antibiotics and antibiotic resistance.

The reactor performance was monitored only for 20 days after the addition of final concentrations of antibiotics to the system. To obtain more insights on the effect of antibiotics on the reactor performance, the analysis should be performed after the steady-state of the reactor is reached. Also, the study of microbial community analysis will give more information on the type of bacteria that is getting affected by the addition of antibiotics.

The process of extraction of antibiotics adsorbed onto the solid phase used in this study showed an error of ~20%. To get the more accurate results of the adsorbed concentrations, the usage of the solid-phase extraction method is recommended. Also, as the concentration of solids used in the adsorption tests was very high compared to the concentration of antibiotics, adsorption was happening in the first 5 minutes, due to which the adsorption kinetics couldn't be studied. To study the kinetics, the solids can be diluted further or the concentration of antibiotics can be increased.

The intermittent feeding degradation batch tests used in this study were meant to replicate the conditions of the reactor to the maximum extent possible. However, there are still a few limitations to this method. The feed used in the batch tests was sodium acetate, whereas the feed of the reactor was synthetic blackwater. This can be included in further studies. Also, the biogas production in the intermittent feeding batch was less than the onetime feeding batch tests. This might be due to a manual error that occurred while adding feed multiple times to the bottles in IF batch tests. To verify this, studying the microbial community of both batch tests can give necessary information.

The TMP removal in the reactor was found to be higher than the SMX, which might be because of the ability of TMP to get adsorbed first and get degraded with time. Hence, the addition of good adsorbents like activated carbon to the reactor can help with the higher removal of antibiotics. Also as SMX and TMP are negatively and positively charged antibiotics respectively, usage of ion exchange resins can be a good solution in this case. However, the addition of these will affect the reactor in different ways and should be studied in detail.

In the analysis of ARGs, qPCR was used in this study, which has some limitations. Only a limited number of genes could be studied given the time constraint. Here, using processes like metagenomics can be helpful. A few other most abundant genes should be analyzed to know more about the spreading of ARGs. Also, to study the mechanisms of resistance dissemination, a study of microbial community analysis can be helpful. The presence of genes can also be studied separately on the intracellular and extracellular DNA to gain an understanding of the propagation of resistant genes. As the removal of ARGs is high in AnMBR, using the post-treatment step might help in producing high-quality effluent that can be reused for potable purposes. Also, as the antibiotic adsorbed onto sludge was negligible in the reactor, the sludge wasted can also be used further for agricultural purposes if the impact of bacteria can be eliminated.

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Appendix A (Information on methodology)

Table 15 Micronutrient composition of feed

Micronutrient Solution Compound	Value	Unit
$FeCl_3.6H_2O$	1000	mg/L
$CoCl_2.6H_2O$	1000	mg/L
$MnCl_2.4H_2O$	250	mg/L
$CuCl_2.2H_2O$	15	mg/L
$ZnCl_2$	25	mg/L
H_3BO_3	25	mg/L
$(NH_4)_6Mo_7O_{24}.4H_2O$	45	mg/L
$Na_2SeO_3.H_2O$	50	mg/L
$NiCl_2.6H_2O$	25	mg/L
EDTA	500	mg/L
HCl 36%	0.5	mL/L
Resazurin sodium salt	250	mg/L
Yeast extract	1000	mg/L

Table 16 Feed composition

Feed Composition: Macronutrients	Value	Unit
Urea	1	g/L
Ammonium chloride	0.8	g/L
Sodium acetate trihydrate	2.6	g/L
Ovalbumin	0.18	g/L
Magnesium sulphate heptahydrate	0.072	g/L
Potassium phosphate monobasic	0.2	g/L
Calcium chloride dihydrate	0.14	g/L
Cellulose	1.5	g/L
Milk powder	0.6	g/L
Yeast extract	0.5	g/L
Sunflower oil	2	mL/L
Micronutrients	10.64	mL/L
Humic and Fulvic acid	0.2	mL/L

Table 17 Membrane characteristics

Membrane characteristics		
Parameter	**Value**	**Unit**
Pore size	30	nm
Type	Tubular, inside out	
Brand	Pentair	
Diameter	5.2	mm
Length	64	cm
Cross sectional area	2.10E-05	m^2
Membrane area	0.01	m^2
Cross flow velocity	0.6	$m.s^{-1}$

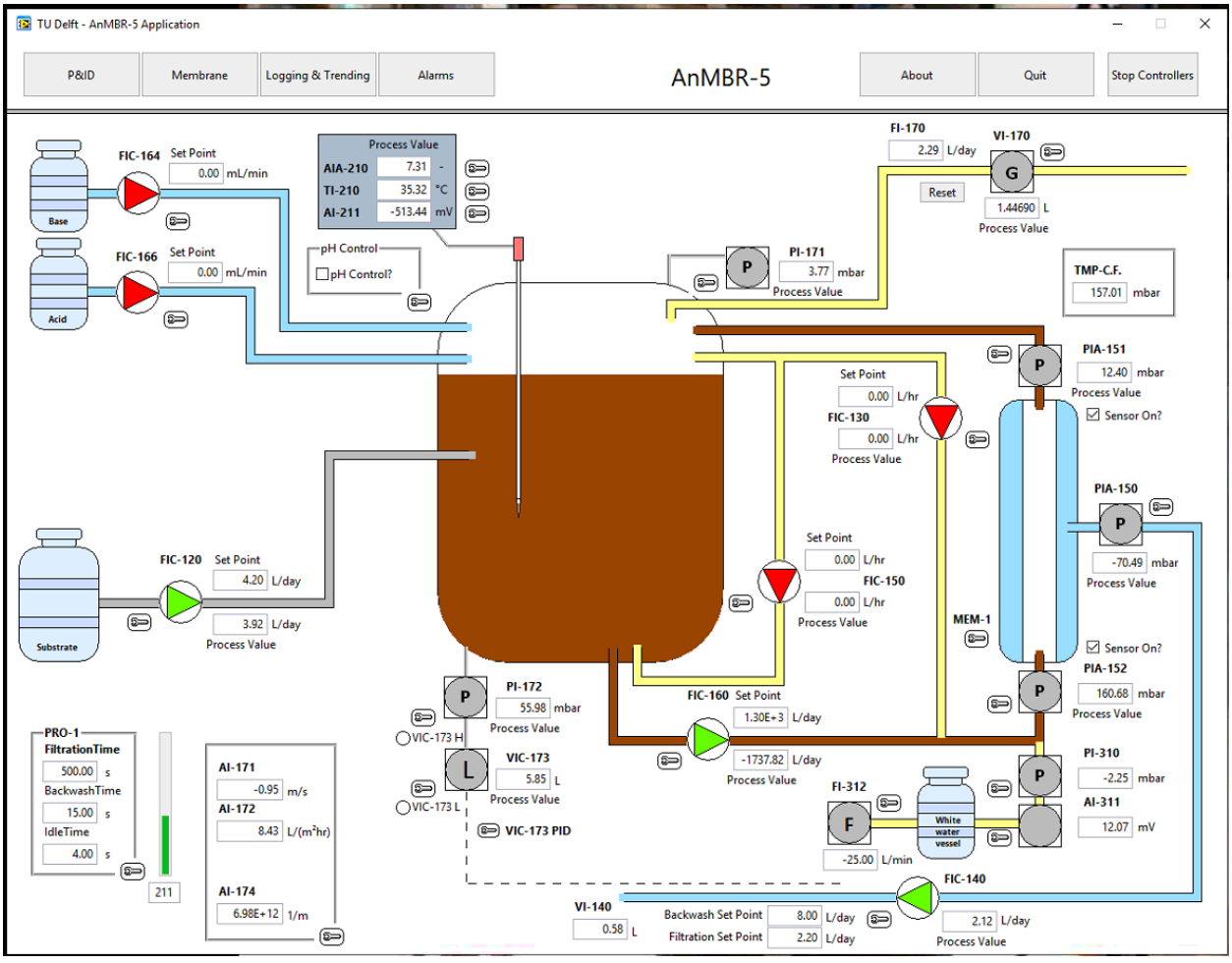


Figure 18 Computer interface of the reactor

COD and Nutrients analysis

The COD of influent and sludge were measure using *Hach Lange's* LCK014 COD test kits with the range 1000-10000 mg COD/L. The effluent COD was measured using *Hach Lange's* LCK514 COD test kits with the range 15-150 mg COD/L.

Nutrients of feed, sludge, and effluent tested were ammonia, nitrate, total nitrogen, phosphate, and sulphate using *Hach Lange's* kits of LCK 303, 339, 338, 350, 153 respectively. After following the test procedure mentioned in the kits accordingly, the samples were analyzed in *Hach Lange's* DR 3900 spectrometer. The dilution factor used for the analysis of NH_4 – N and Total N of the samples was 20, and the dilution factor for the analysis of PO_4 – P, and SO_4^{-2} was five.

Preparation of internal standards for LCMS

An internal standard for each antibiotic was prepared using the isotopes of the compound to ensure it represents the behavior of the particular antibiotic accurately. The equipment could separate these two compounds and quantify the analyte based on the ratio of peak area of the analyte to the internal standard.

The internal standards for sulfamethoxazole and trimethoprim used were sulfamethoxazole-D4 and trimethoprim-D9. Trimethoprim-D9 is also used as the internal standard for ciprofloxacin and ampicillin. The 10 mg/L internal standard stock solutions of each were prepared by mixing them separately in water/acetonitrile. This was further diluted to 100 µg/L and named as "iSTDMix" and was stored at -20 ˚C for further use.

Preparation of calibration curve for LCMS

The calibration line was prepared for each antibiotic to be analyzed manually, and this was considered as an external standard. The calibration curve was used to establish a standard to which the actual sample concentration obtained from MS was then compared. This calibration curve was made by preparing different concentrations of standard solutions shown in Table 18. Initially, stock solutions of stock 1 and stock 2 were prepared from concentrated antibiotic stocks. These stock 1 and 2 were diluted serially with ultrapure water to get the final required concentrations shown in Table 18. All the calibration samples were prepared by adding 10 µL of iSTDMix first, followed by ultrapure water, and lastly, the stock 1 or 2 was added. From these calibration samples, a linear curve was obtained which was then applied to the sample measurement.

Table 18 LC-MS calibration curve

stock name =>	iStd. Mix	stock 1	stock 2	Ultrapure water
stock conc. [µg/L] =>	100	50	0.5	-
Target conc. (µg/L)	V to add (µL)	V to add (µL)	V to add (µL)	V to add (µL)
0	10		0	990
0.0025	10		5	985
0.005	10		10	980
0.01	10		20	970
0.05	10		100	890
0.1	10		200	790
0.5	10	10		980
1	10	20		970
2.5	10	50		940
5	10	100		890
10	10	200		790

Table 19 Primers of selected genes

Genes	F. Primer 5′-3′	R. Primer 5′-3′
sul1	CGCACCGGAAACATCGCTGCAC	TGAAGTTCCGCCGCAAGGCTCG
sul2	TCCGGTGGAGGCCGGTATCTGG	CGGGAATGCCATCTGCCTTGAG
dfrA1	TTCAGGTGGTGGGGAGATATAC	TTAGAGGCGAAGTCTTGGGTAA
intl1	GATCGGTCGAATGCGTGT	GCCTTGATGTTACCCGAGAG
16s rRNA	ACTCCTACGGGAGGCAGCAG	ATTACCGCGGCTGCTGG

Table 20 Amplification conditions for selected genes

Gene	Amplification conditions
sul1	5 min at 95°C, 40 cycles of 15 s at 95°C, 30 s at 65°C
sul2	5 min at 95°C, 40 cycles of 15 s at 95°C, 30 s at 61°C
dfrA1	7 min at 95°C, 40 cycles of 10 s at 95°C, 30 s at 60°C
intl1	5 min at 95°C, 40 cycles of 15 s at 95°C, 30 s at 60°C
16s rRNA	5 min at 95°C, 40 cycles of 15 s at 95°C, 30 s at 60°C

Bottle No.	Sample Type	Aeration (mlair/bottle/d)	Total Volume (ml)	Inoculum Volume (mL)	Inoculum VS (g)	Substrate C VS (g)	Substrate C mass (g)	Antibiotics concentration (µg/L)	Expected methane (ml of methane)
1	negative control	0	99	66.00	0.22	0	0	0	
2	negative control	0	99	66.00	0.22	0	0	0	
3	negative control	0	99	66.00	0.22	0	0	0	
4	positive control	0	99	66.00	0.22	0.11	0.51	0	84.10
5	positive control	0	99	66.00	0.22	0.11	0.51	0	84.10
6	positive control	0	99	66.00	0.22	0.11	0.51	0	84.10
7	TMP 150	4.2	99	66.00	0.22	0.11	0.51	150.00	84.10
8	TMP 150	4.2	99	66.00	0.22	0.11	0.51	150.00	84.10
9	TMP 150	4.2	99	66.00	0.22	0.11	0.51	150.00	84.10
10	SMX+TMP 150	4.2	99	66.00	0.22	0.11	0.51	150.00	84.10
11	SMX+TMP 150	4.2	99	66.00	0.22	0.11	0.51	150.00	84.10
12	SMX+TMP 150	4.2	99	66.00	0.22	0.11	0.51	150.00	84.10
13	CIP+AMP 10	4.2	99	66.00	0.22	0.11	0.51	10.00	84.10
14	CIP+AMP 10	4.2	99	66.00	0.22	0.11	0.51	10.00	84.10
15	CIP+AMP 10	4.2	99	66.00	0.22	0.11	0.51	10.00	84.10
16	CIP+AMP 50	4.2	99	66.00	0.22	0.11	0.51	50.00	84.10
17	CIP+AMP 50	4.2	99	66.00	0.22	0.11	0.51	50.00	84.10
18	CIP+AMP 50	4.2	99	66.00	0.22	0.11	0.51	50.00	84.10
19	CIP+AMP 150	4.2	99	66.00	0.22	0.11	0.51	150.00	84.10
20	CIP+AMP 150	4.2	99	66.00	0.22	0.11	0.51	150.00	84.10
21	CIP+AMP 150	4.2	99	66.00	0.22	0.11	0.51	150.00	84.10

Figure 19 Matrix of biodegradation test with onetime feeding

Bottle No.	Sample Type	Substrate addition	Aeration (mlair/bottle/d)	Total Volume (ml)	Inoculum Volume (mL)	Inoculum VS (g)	Substrate C mass (g/d)	Aeration %VS	Expected methane (ml of methane)
1	negative control	-	0	99	66.00	0.20	0	0	0
2	negative control	-	0	99	66.00	0.20	0	0	0
3	negative control	-	0	99	66.00	0.20	0	0	0
4	positive control 1	one time	0	99	66.00	0.20	0.4656	0	76.7
5	positive control 2	one time	0	99	66.00	0.20	0.4656	0	76.7
6	positive control 3	one time	0	99	66.00	0.20	0.4656	0	76.7
7	positive control 4	one time	3.8	99	66.00	0.20	0.4656	2.02	76.7
8	positive control 5	one time	3.8	99	66.00	0.20	0.4656	2.02	76.7
9	positive control 6	one time	3.8	99	66.00	0.20	0.4656	2.02	76.7
10	positive control 7	daily	3.8	99	66.00	0.20	0.0931	2.02	76.7
11	positive control 8	daily	3.8	99	66.00	0.20	0.0931	2.02	76.7
12	positive control 9	daily	3.8	99	66.00	0.20	0.0931	2.02	76.7
13	SMX+TMP 150	daily	3.8	99	66.00	0.20	0.0931	2.02	76.7
14	SMX+TMP 150	daily	3.8	99	66.00	0.20	0.0931	2.02	76.7
15	SMX+TMP 150	daily	3.8	99	66.00	0.20	0.0931	2.02	76.7
16	CIP+AMP 150	daily	3.8	99	66.00	0.20	0.0931	2.02	76.7
17	CIP+AMP 150	daily	3.8	99	66.00	0.20	0.0931	2.02	76.7
18	CIP+AMP 150	daily	3.8	99	66.00	0.20	0.0931	2.02	76.7

Figure 20 Matrix of biodegradation test with intermittent feeding

Appendix B (Results of performance of reactor)

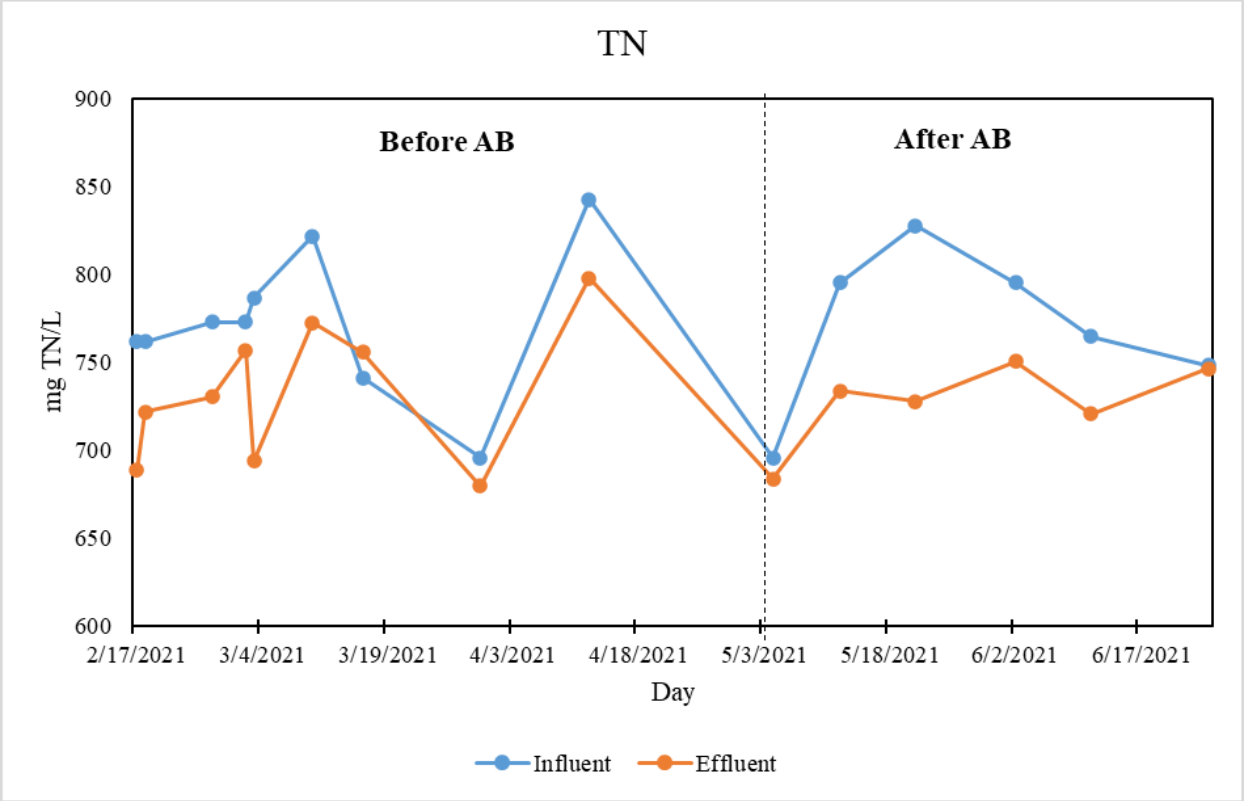


Figure 21 Total nitrogen in reactor

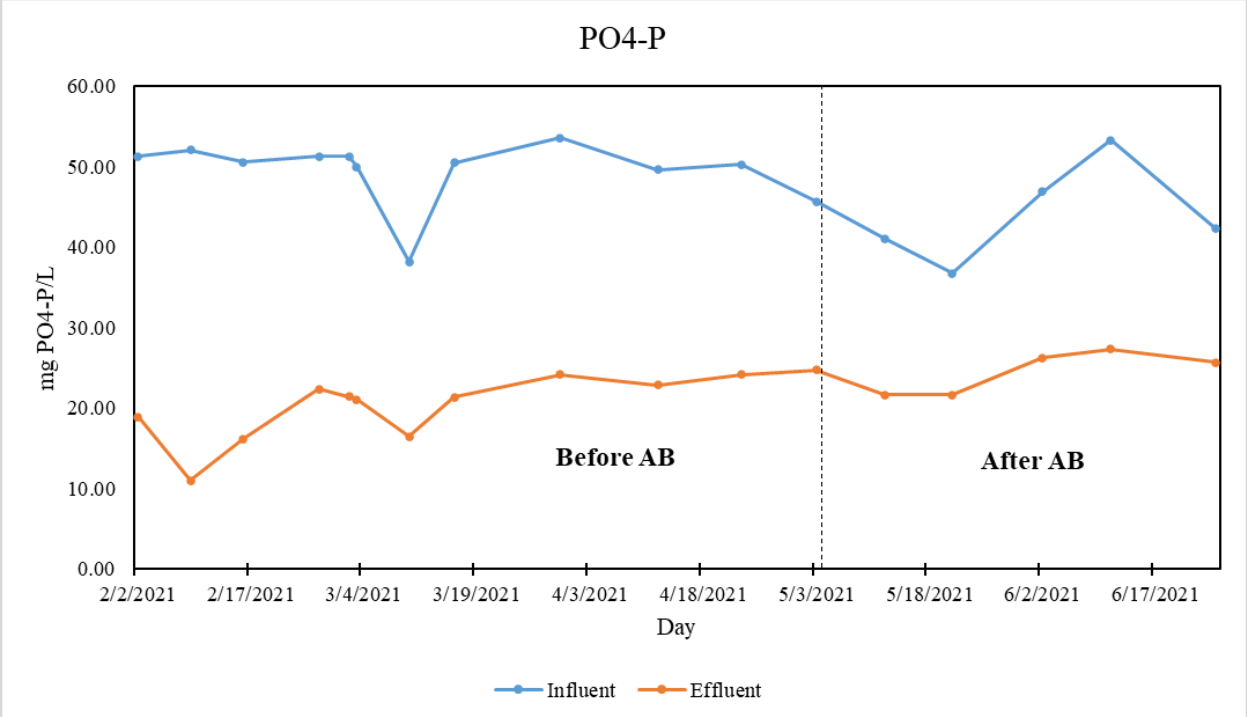


Figure 22 Removal of phosphate in reactor

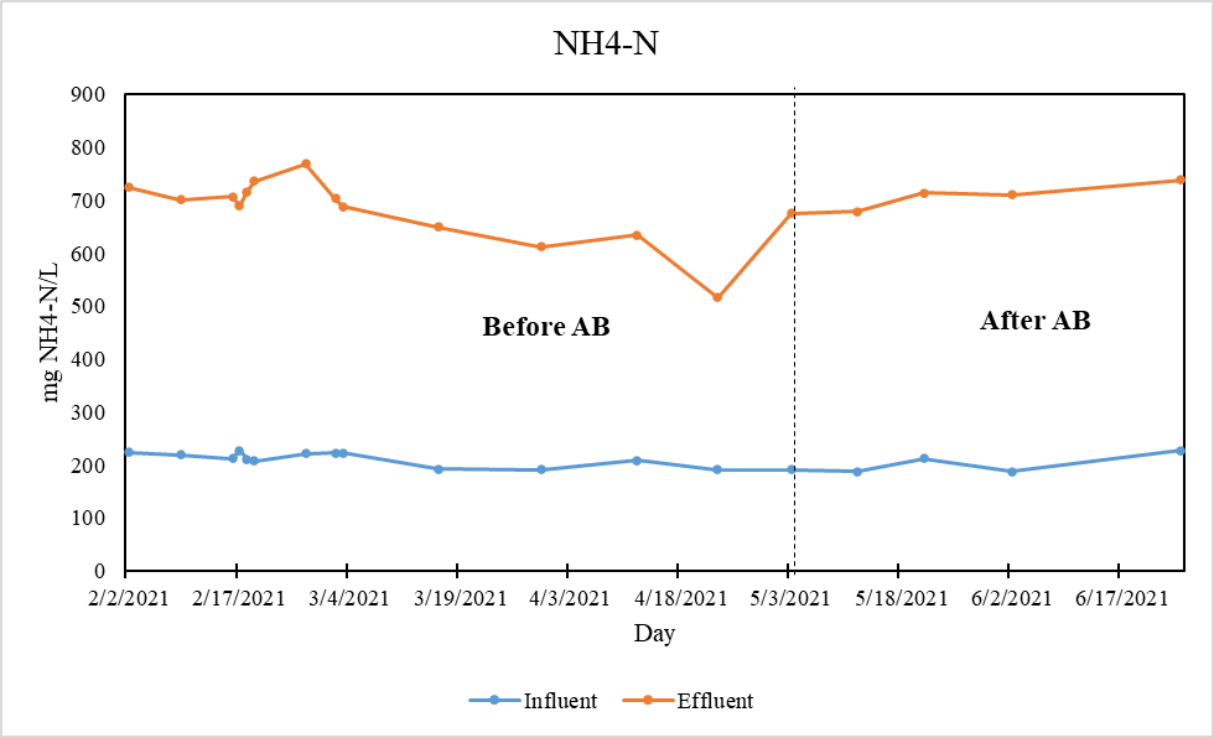


Figure 23 Removal of Ammonia in reactor

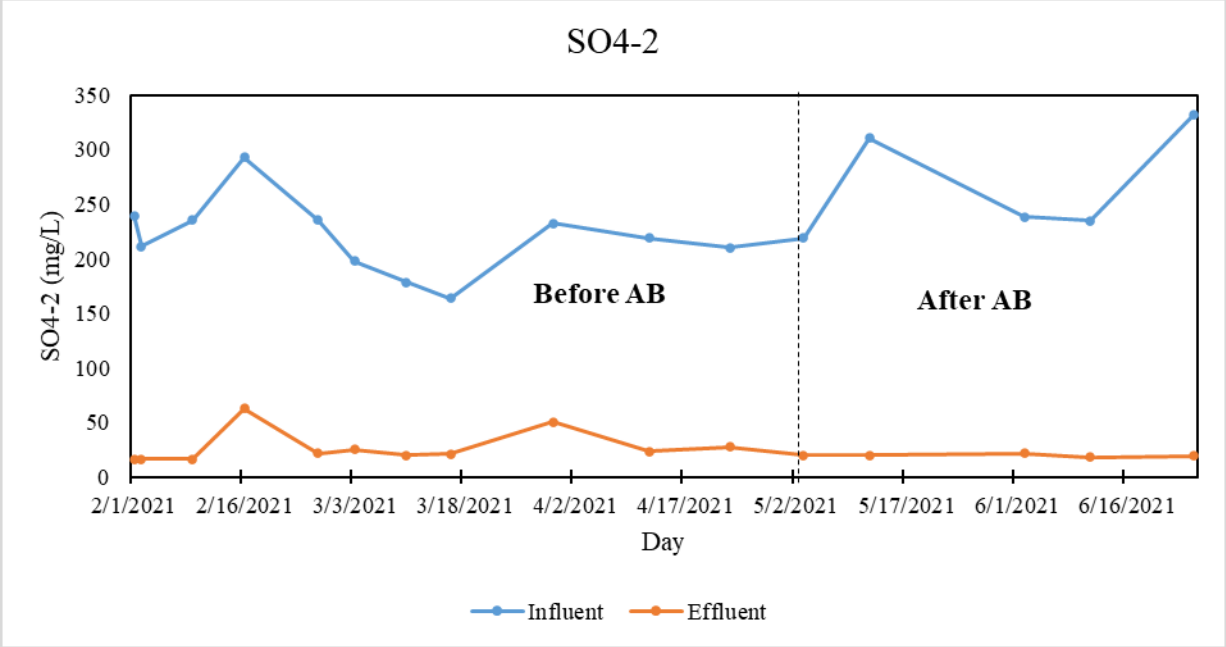


Figure 24 Removal of sulphate in reactor

Table 21 Variation in biomass properties after antibiotics addition

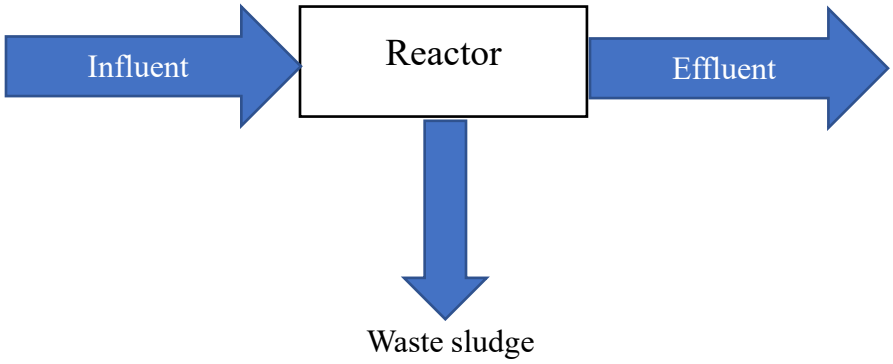
Property	**Before AB**	**After AB**
Sludge COD mg/L	4512±1207	4477±1551
TS g/L	7.13±1.7	7.80±1.5
VS g/L	3.31±0.9	3.45±1.1
TSS g/L	5.31±1.5	5.27±1.3
VSS g/L	3.17±0.82	3.17±0.78
ORP mV	-537±19	-536±11
pH	7.77±0.14	7.56±0.12

Table 22 Concentration of VFA

VFA	**Before AB**	**After AB**
Acetic acid (mg/L)	3.8±4.2	8.4±7.7
Propionic acid (mg/L)	2.48	4.5±2.7

Appendix C (SMX and TMP removal)

Antibiotics Mass Balance in the reactor



$$AB_{inf} = AB_{sorption} + AB_{WS} + AB_{Eff} + Biodegradation \quad (\mu g/d)$$

Sorption to biomass = q × Solids × $\text{Volume}_{\text{reactor}}$

AB waste sludge = q × Solids × $\text{Volume}_{\text{waste sludge}}$

q = Antibiotic sorbed onto sludge (µg/g)

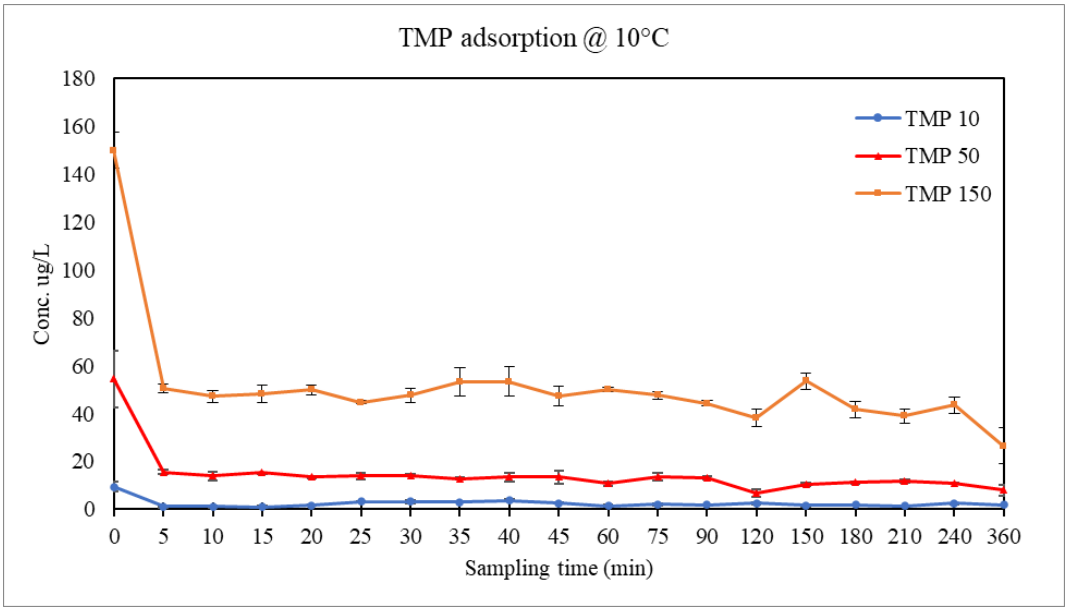


Figure 25 Adsorption of TMP at 10°C

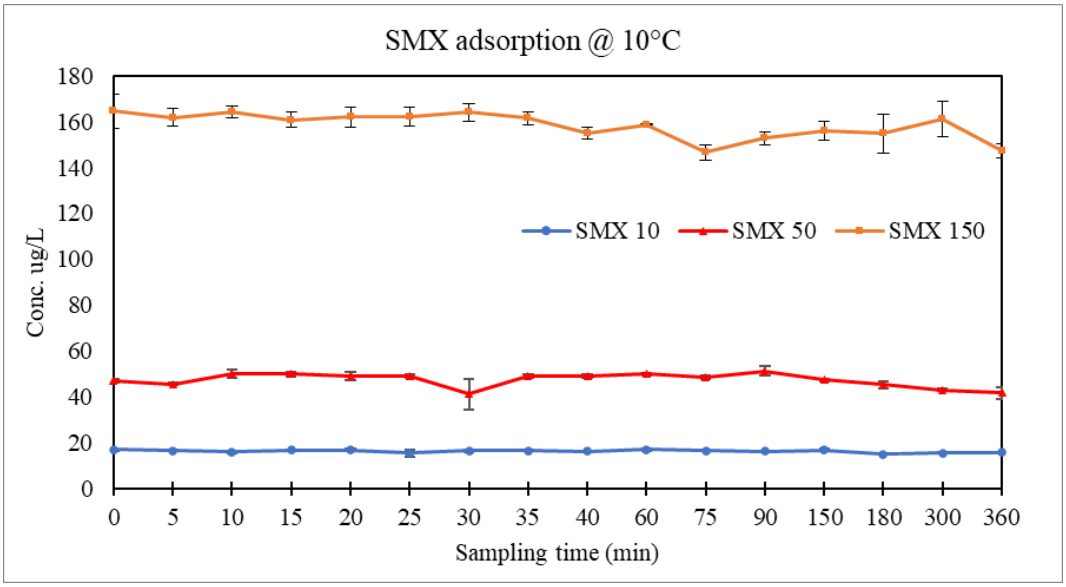


Figure 26 Adsorption of SMX at 10°C

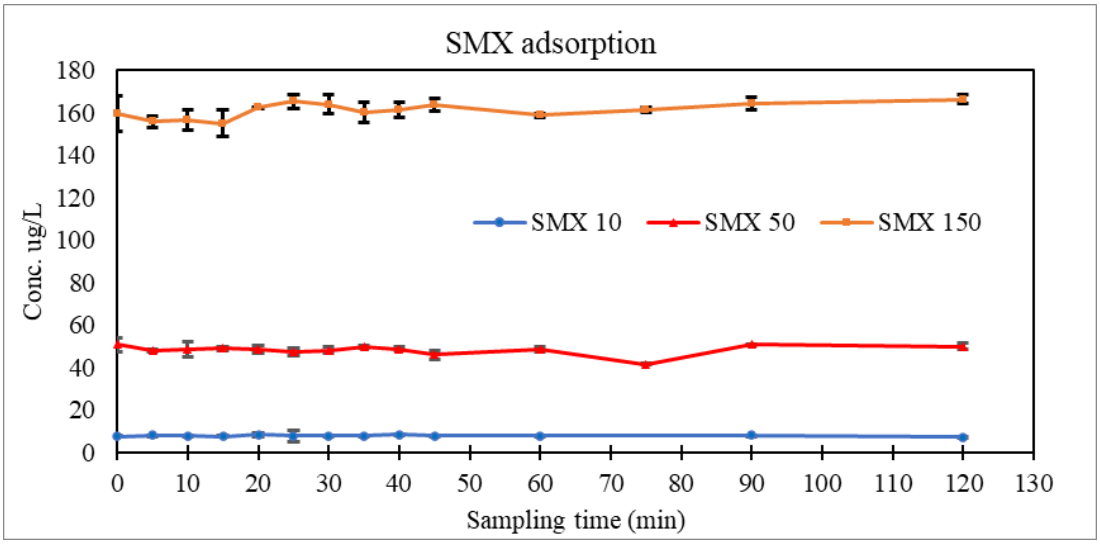


Figure 27 Adsorption of SMX at 37°C

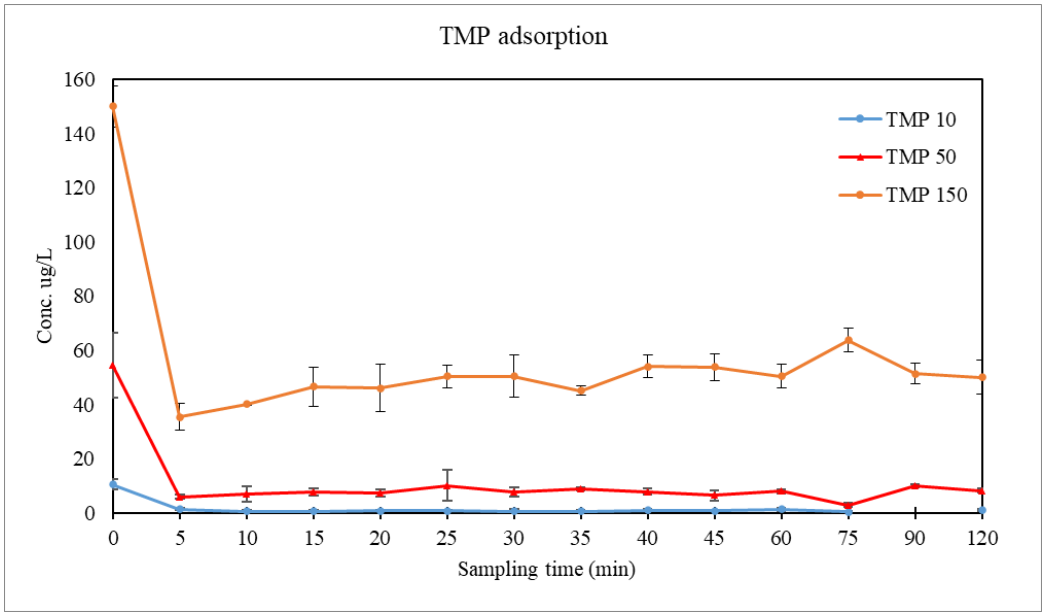


Figure 29 Adsorption of TMP at 37°C

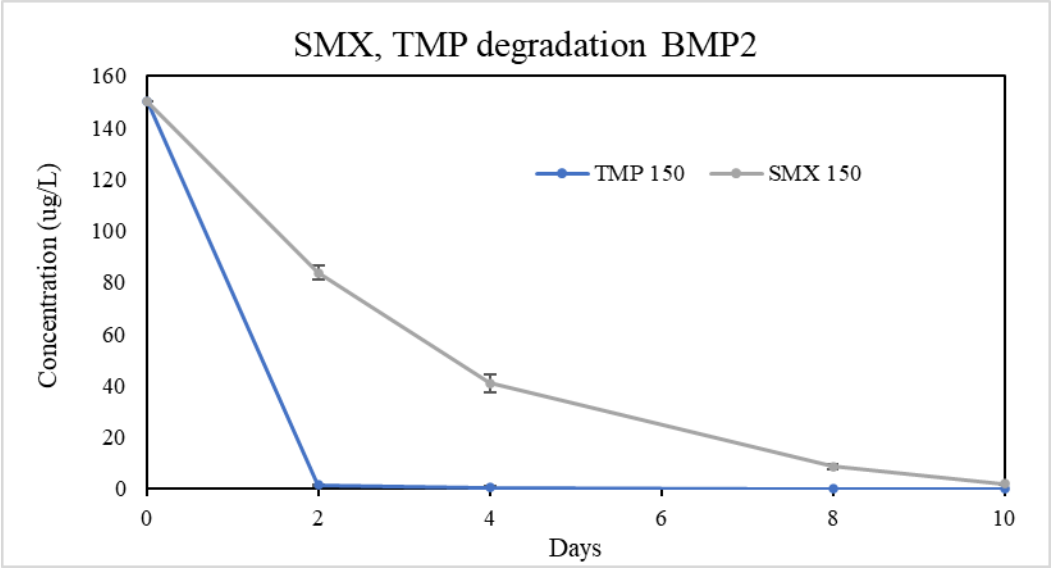


Figure 28 Degradation of SMX and TMP via intermittent feeding batch tests

Table 23 Correlations among ARB, ARG and concentrations of antibiotics

	sul1	**sul2**	**intI1**	**SMX RB**	**TMP RB**
sul2	-0.27				
intI1	0.10	***0.73***			
SMX RB	***0.93***	-0.39	-0.05		
TMP RB	***0.91***	-0.42	-0.13	***0.99***	
SMX conc	***0.86***	-0.52	-0.20	***0.89***	***0.89***
TMP conc	***0.83***	0.09	***0.99***	0.37	0.48

Appendix D (Effect of CIP and AMP on biomass)

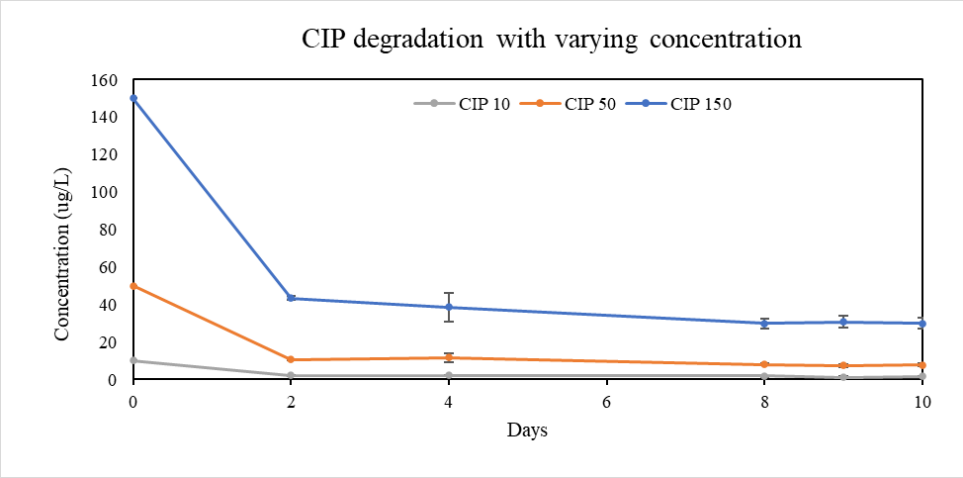


Figure 30 Removal CIP at varying concentrations in BD test 1

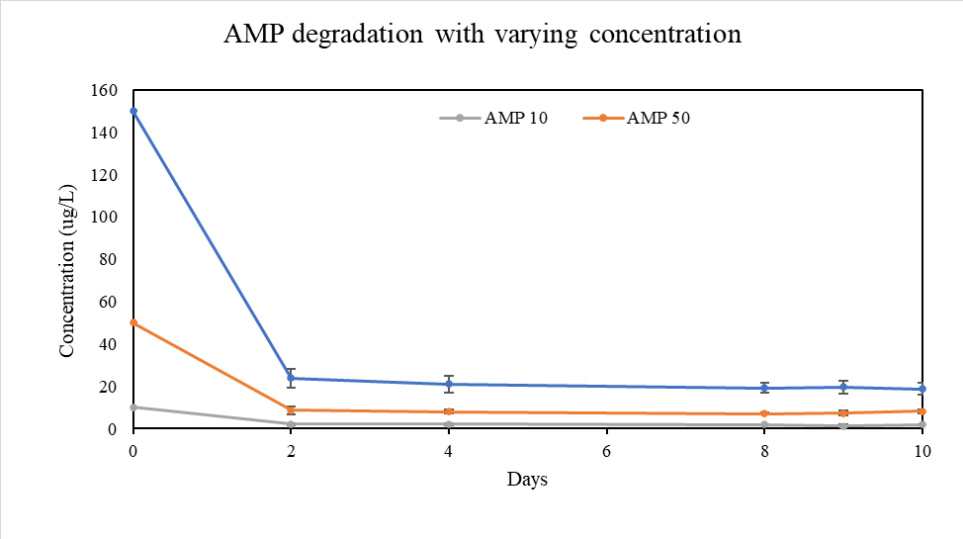


Figure 31 Removal AMP at varying concentrations in BD test 1

The cumulative methane production in the intermittent feeding (IF) batch tests are shown in Figure 32. In addition to the batch tests with antibiotics, two types of positive controls (PC): onetime feeding and intermittent feeding were also included in this test. The comparison of the biomethane potentials (BMP) between the PC and the batch with CIP and AMP helped to know the effect of the antibiotics on the biomass. BMP is expressed in terms of dry volume of methane under STP (273K and 101 kPa) conditions per volume of VS added in the batch. The calculated BMPs for all the batches are expressed in terms of the percentage of expected theoretical BMP (Table 24).

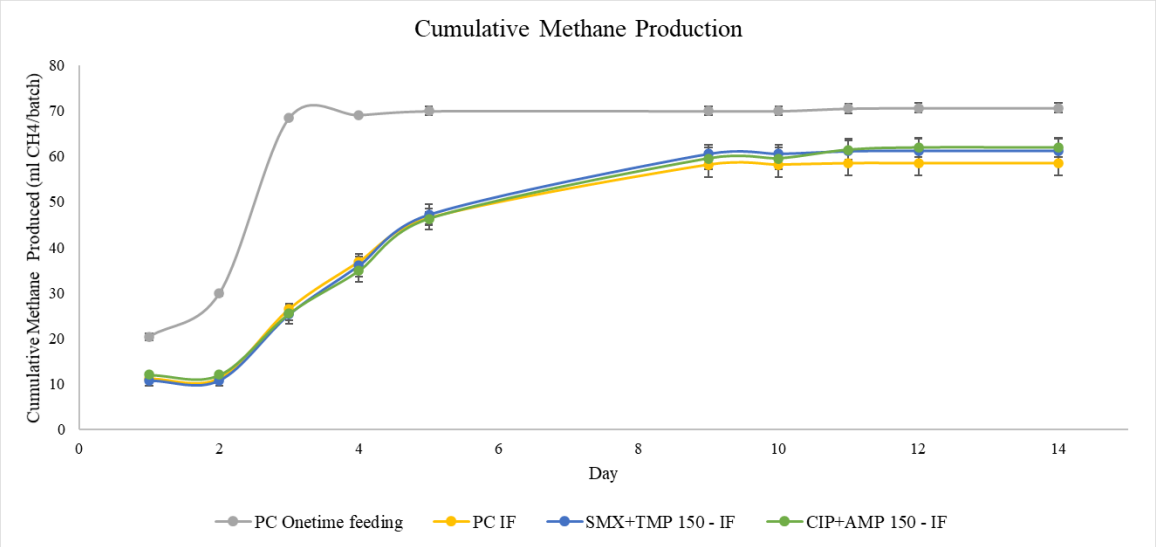


Figure 32 Cumulative methane production in intermittent feeding batch test

It can be seen that the BMP of PC-IF is around 78% and is less than the BMP of PC onetime feeding which has a value of 92%. The main reason for this might be the manual error that occurred while injecting the substrate into the bottles daily, which can also be seen from the high relative standard deviations (RSD) in Table 24. However, it can be seen that the BMP of PC-IF and the bottle with CIP, AMP were almost similar with a value of around 81%. Hence, it can be concluded that the antibiotics CIP and AMP at the given concentrations have negligible effects on the biomass.

Table 24 BMP of various batches of intermittent feeding

	%BMP to theoretical	**RSD %**
PC Onetime feeding	92.1	1.55
PC - IF	77.4	4.34
CIP+AMP150 µg/L - IF	81.8	3.40

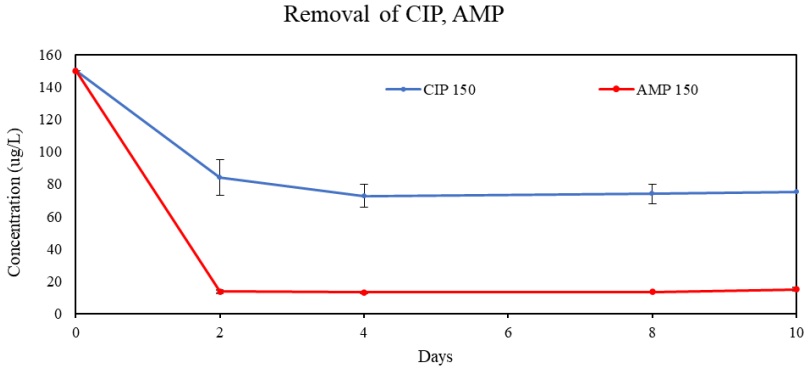


Figure 33 Removal of CIP and AMP in intermittent feeding batch tests