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Antibiotic Resistance genes dynamics and operation performance of AnMBRs under Amoxicillin/Flucloxacillin Exposure

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Abstract

Anaerobic membrane bioreactors (AnMBRs) offer a promising solution for wastewater treatment, combining anaerobic digestion with membrane filtration to efficiently remove pollutants and mitigate the spread of antibiotic resistance genes (ARGs). The impact of antibiotic mixtures on AnMBR performance and ARG profiles remains crucial to investigate due to their prevalence in various environments. This study examined the effects of an amoxicillin and flucloxacillin mixture on key operational parameters in an AnMBR system. The AnMBR demonstrated resilience to the antibiotic mixture, maintaining a high average chemical oxygen demand (COD) removal efficiency (91 ± 4.5%) and stable biogas production (713 ± 150 L/d) even at elevated antibiotic concentrations (250 µg/L). However, antibiotic presence significantly influenced ARG profiles within the biomass and effluent, highlighting the complex interplay between antibiotic exposure and resistance gene dynamics. These findings emphasize the importance of understanding and optimizing AnMBR performance under antibiotic pressure to mitigate the spread of antibiotic resistance while ensuring effective wastewater treatment and resource recovery.

Keywords: *Antibiotic resistance genes (ARGs); Anaerobic membrane bioreactors (AnMBRs); Wastewater treatment; Amoxicillin; Flucloxacillin; Antibiotic mixture.*

1. Introduction

Anaerobic membrane bioreactors (AnMBRs) represent a cutting-edge approach to wastewater treatment, harnessing the combined power of anaerobic digestion and membrane filtration [1–3]. During anaerobic digestion, microorganisms break down organic matter in the absence of oxygen, producing biogas as a valuable byproduct. On the other hand, membrane filtration utilizes semi-permeable membranes to separate solids and contaminants from water efficiently. This synergistic integration in AnMBR systems ensures the efficient treatment of municipal wastewater and unlocks the potential for resource recovery through biogas production, thereby contributing to the circular economy and promoting sustainable practices [1–3]. Studies have demonstrated the effectiveness of AnMBRs in treating municipal wastewater, showcasing their ability to achieve high levels of organic matter removal and biogas production while maintaining stable operation, even under fluctuating organic loading rates [3]. In addition to their wastewater treatment and energy generation capabilities, AnMBRs have emerged as a powerful tool in mitigating the growing threat of antibiotic resistance [4–6]. By significantly reducing the release of Antibiotic Resistance Genes (ARGs) into the environment, AnMBRs play a crucial role in safeguarding public health. The membrane within these systems acts as a robust barrier, effectively retaining biomass, including microorganisms and larger mobile genetic elements (MGEs) that may harbor antibiotic resistance genes. This retention mechanism substantially curtails the dissemination of antibiotic resistance through treated effluent, establishing AnMBRs as a sustainable and effective solution in combating antibiotic resistance proliferation [4,5]. Research has further highlighted the capacity of AnMBRs to effectively treat antibiotic-laden wastewater, particularly from pharmaceutical sources, with promising results in terms of both antibiotic removal and ARG reduction [5,6].

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β-lactam antibiotics, including amoxicillin and flucloxacillin, are extensively used in various sectors, particularly poultry and aquaculture. This widespread use has led to significant β-lactam contamination in water bodies, as evidenced by high levels detected in river water in regions with intensive agricultural practices [7]. Amoxicillin, a broad-spectrum penicillin, is frequently detected in wastewater due to its extensive use in both human and veterinary medicine [5,8]. Flucloxacillin, a penicillinase-resistant penicillin, is also commonly found in wastewater, particularly from hospital effluents [6]. The presence of β-lactams like amoxicillin and flucloxacillin in wastewater exerts selective pressure on microbial communities, potentially leading to an increase in β-lactamase ARGs. This phenomenon has been observed in studies on AnMBR treatment of poultry slaughterhouse wastewater [9]. Moreover, the prevalence of extended-spectrum β-lactamase (ESBL)-producing bacteria, such as *E. coli* and *Klebsiella pneumoniae*, harboring bla gene variants (e.g., blaNDM, blaSHV, blaTEM), has been reported in various regions, further highlighting the concern of β-lactam resistance dissemination [10–12].

While the clinical implications of antibiotic resistance have garnered significant attention, it is crucial to acknowledge the broader ecological context in which this phenomenon unfolds. Wastewater treatment plants (WWTPs), often overlooked, play a critical role in disseminating antibiotic-resistant genes into the environment. These facilities serve as reservoirs for ARGs originating from natural and human-induced processes, including releasing antibiotic residues and resistant bacteria from various sources such as hospitals, agricultural operations, and households [6,13,14]. In aquatic environments, WWTPs facilitate the transfer of these genes among diverse bacterial communities, creating a complex web of interactions that contribute to the growing global health threat posed by antibiotic resistance [6,13,14]. The presence of mercury and silver resistance genes, often associated with ARGs, further underscores the interconnectedness of these environmental contaminants and emphasizes the need for comprehensive monitoring and mitigation strategies within WWTPs [14].

The public health implications of antibiotic resistance are severe and widespread. In the United States alone, an estimated 20 billion dollars in excess healthcare costs and an additional 8 million hospital days are attributed to antibiotic-resistant infections annually [4]. The escalating prevalence of resistance, driven by the selective pressures exerted by antimicrobial compounds and facilitated by efficient vertical and horizontal gene transfer mechanisms among bacteria, necessitates urgent action [4,15–21]. Studies have revealed that WWTPs often discharge treated effluent containing altered concentrations of various ARGs, sometimes exceeding levels found in the influent wastewater [4,22]. This alarming observation, also supported by research demonstrating increased ARG abundance in response to combined antibiotic exposure [21], underscores the potential of WWTPs to act as amplifiers and disseminators of antibiotic resistance, highlighting the critical need for targeted interventions to mitigate this public health crisis [4].

Recent research has shed light on the complex interplay between antibiotic mixtures and the profiles of ARGs within microbial communities. Studies have demonstrated that the combined selective pressures exerted by multiple antibiotics can enrich and disseminate broader spectrum of resistance genes compared to individual antibiotics [15–19,21]. These findings emphasize the importance of understanding the dynamics of resistance gene expression in response to antibiotic cocktails, which are frequently encountered in wastewater treatment plants and other environments, such as agricultural soils exposed to antibiotic-laden manure [23,24]. Such knowledge is essential for developing effective strategies to combat the spread of antibiotic resistance and protect public health [15–19,21].

The escalating global water scarcity crisis, driven by factors such as population growth, uncontrolled economic growth, the need to raise living standards, climate change, and escalating water pollution, necessitates innovative solutions like wastewater reuse [25]. Wastewater reuse is a cornerstone of the circular economy model, promoting resource recovery and sustainable water management. However, the presence of contaminants, including antibiotics, in wastewater raises valid concerns for both environmental and human health [5,6]. Antibiotics, extensively used in medicine, agriculture, and animal husbandry, find their way into wastewater streams, often in substantial concentrations [5]. For instance, the excessive use of antibiotics in animal husbandry leads to the excretion of unmetabolized antibiotic residues in animal waste, with concentrations ranging from milligrams to grams per kg [24]. Similarly, pharmaceutical wastewater can contain antibiotic concentrations exceeding 20 mg/L [8]. Discharging such wastewater into the environment inevitably impacts the natural microbial communities, fostering the proliferation of antibiotic-resistant bacteria (ARBs) and ARGs [6,20,21].

The continuous exposure of bacteria to antibiotics within wastewater treatment systems creates persistent selective pressure, favoring the survival and proliferation of those possessing resistance mechanisms changes of antibiotic resistance genes and bacterial communities in the advanced biological wastewater treatment [20,21,26]. Resistance genes can be found both intracellularly, within bacterial cells, and extracellularly, as free DNA in the environment [27]. Bacteria employ diverse strategies to evade the effects of antibiotics. These strategies include the production of enzymes that degrade antibiotics (e.g., β-lactamase enzymes in Enterobacteriaceae that counteract penicillin) [28], the expression of efflux pumps that expel antibiotics from the cell [26], and modifications to the antibiotic target sites within the bacterial cell [26,28]. These mechanisms, coupled with the ability of bacteria to transfer resistance genes vertically through replication and horizontally through conjugation, transduction, and transformation, contribute to the rapid spread of antibiotic resistance within and beyond WWTPs [29–32].

Anaerobic membrane bioreactors (AnMBRs), which integrate anaerobic digestion with membrane filtration, offer a

promising solution for mitigating the release of ARGs and other contaminants from wastewater. The anaerobic digestion process facilitates the degradation of organic matter and energy recovery and creates an environment that may be less conducive to the proliferation of certain antibiotic-resistant bacteria [1,2]. The membrane component further enhances the removal of ARGs and ARBs by physically separating them from the treated effluent. Additionally, AnMBRs possess several advantages over conventional biological wastewater treatment methods, including enhanced energy recovery through biogas production, improved biodegradation of organic pollutants, extended sludge retention times (SRTs), and superior effluent quality [1,2].

The primary objective of the present study is to investigate the intricate relationship between antibiotic mixtures and AnMBR performance, specifically focusing on the effects of an amoxicillin and flucloxacillin mixture on key operational parameters such as COD removal efficiency, biogas production, and the fate of the antibiotics within the system. While previous studies have explored the impact of individual antibiotics or antibiotic classes on AnMBRs, there remains a knowledge gap in understanding how these systems respond to mixtures of antibiotics, which are commonly encountered in real-world wastewater. Furthermore, the long-term effects of antibiotic exposure on AnMBR performance and ARG profiles have not been fully elucidated. By elucidating the impacts of these commonly used antibiotics on the performance of AnMBRs, this research seeks to contribute valuable insights to the ongoing efforts to optimize wastewater treatment processes, mitigate the spread of antibiotic resistance, and protect both human and environmental health.

2. Material and methods

2.1. AnMBR Configuration and Operation

The laboratory-scale AnMBR system (Figure 1), with a working volume of 10 liters, incorporated four flat-sheet silicon carbide (Ceramic) microfiltration membranes with a 0.1 µm pore size (Cembrane A/S, Denmark). The reactor operated at 25°C. These membranes, positioned on either side of the reactor with a central space for the mixer, provided a total effective filtration area of 0.02 m^2 [33]. The system was operated at a hydraulic retention time (HRT) of 16 hours, corresponding to a flow rate of 625 mL/h and a membrane flux of 15.625 $L/m^2/h$. Influent wastewater was delivered to the reactor using a peristaltic pump, and another pump facilitated permeate withdrawal through the ceramic membranes. Two float switches maintained a stable liquid level within the reactor. The primary float switch controlled the influent flow, while the secondary switch acted as a safety mechanism to prevent overfilling and leakage.

A biogas recirculation system was implemented to enhance membrane cleaning and mitigate fouling. A biogas pump with a capacity of 12 L/min delivered 3 L/min to each of the two diffusers located beneath the membranes. This recirculation occurred for one minute every ten minutes. Each membrane module's transmembrane pressure (TMP) was monitored using a pressure transducer, and the transmembrane pressure (TMP) was kept below -0.8 bar.

Sludge was not wasted except for sampling purposes, leading to a solids retention time (SRT) of about 100 days by the end of the experiment.

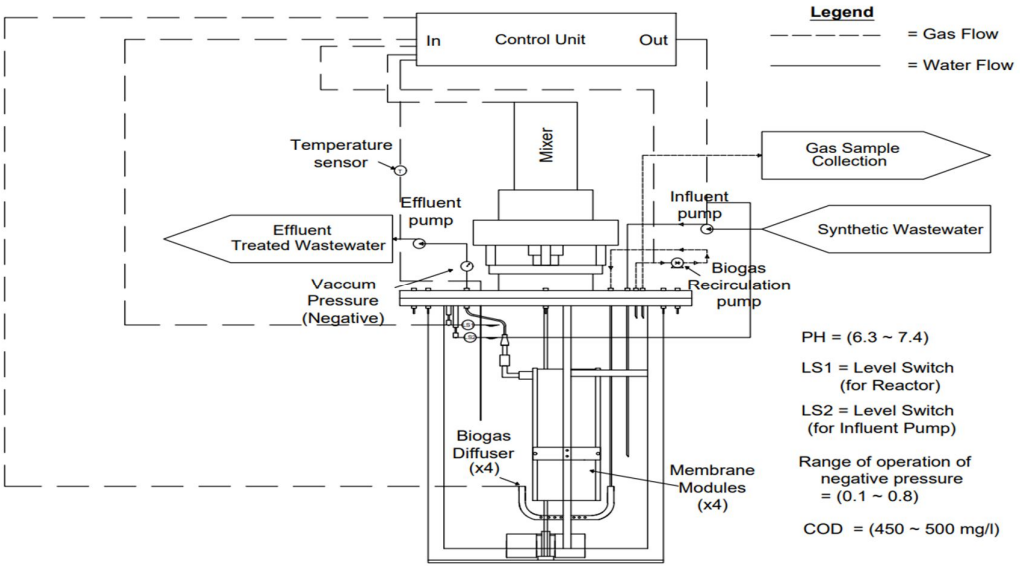

Fig. 1. Schematic diagram for the lab-scale anaerobic membrane bioreactors (AnMBR)

2.2. Synthetic Wastewater and Antibiotic Mixture

The AnMBR system was fed with synthetic wastewater, and formulated to maintain a constant chemical oxygen demand (COD) concentration of 500 mg/L throughout the experiment [33]. This approach allowed for precise control over antibiotic introduction, eliminating the potential influence of background contaminants present in real wastewater.

The synthetic wastewater was prepared according to a predetermined composition (Table 1), with a concentrated stock solution stored at 4°C. Before each use, tap water diluted an aliquot of the stock solution to the desired concentration.

An antibiotic mixture, consisting of a 1:1 ratio of amoxicillin and flucloxacillin, was prepared in concentrations of 50, 100, and 250 µg/L, reflecting typical levels found in domestic wastewater and hospital effluents [33]. This mixture, sourced from a commercially available drug (Flumox, EIPICO, Egypt), was stored at 4°C until use.

The AnMBR was initially operated without antibiotics to establish a steady-state performance, characterized by an effluent COD removal efficiency of up to 90%. Subsequently, the antibiotic mixture was introduced into the influent wastewater in three distinct phases, each lasting 10 days. The antibiotic concentrations were incrementally increased from 50 to 100, then to 250 µg/L.

Throughout the experiment, the AnMBR's performance was comprehensively monitored. Key parameters, including mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), COD, biogas production, and methane content in the biogas were regularly measured to assess the system's operational stability, treatment efficacy, and biogas generation potential [33]. Biogas produced in the reactor were collected in a 1-liter biogas sampling bag from the reactor headspace, and the time taken to fill the bag was recorded to estimate biogas production.

Table 1. Composition of Synthetic Wastewater

Inorganics	Conc.(g/l)	Organics	Conc. (g/l)
NH_4Cl	0.15	Milk Powder	1.5
Na_2SO_4	0.15	Peptone	0.15
$NaHCO_3$	0.3	Starch	1.5
K_3PO_4	0.015	Yeast	0.6
		$C_2H_3NaO_2$	0.5
		Urea	0.55

2.3. Sample DNA Extraction

AnMBR biomass and effluent samples were collected before antibiotic application (at the end of the start-up period) and prior to each increase in antibiotic concentration (every 10 days). For effluent samples, 12 mL was centrifuged, whereas two mL of suspended sludge was centrifuged for biomass samples. Both were centrifuged for 10 min at 14,000 RPM. The samples were first pretreated with lysis buffer, followed by three cycles of freezing and thawing using liquid nitrogen. Following supernatant removal, the lysates were purified using a Genomic DNA Kit (GeneJET ™, Thermo Scientific, USA), and the kit's instructions were followed for DNA purification. The extracted DNA was then quantified, and its quality assessed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Thermo Scientific, USA).

2.4. Quantification of ARGs by qPCR

Conventional Polymerase Chain Reaction (PCR) assays were employed to detect the presence of three beta-lactam resistance genes (*oxa*-1, bla_{TEM}, and bla_{NDM}) in the AnMBR biomass and effluent [3,8,20]. The PCR reactions were carried out using a 2x master mix (amaR OnePCR™, GeneDireX, Inc.) in a total 20 µl reaction volume. The PCR program included initial denaturation at 95°C, followed by 30 cycles of denaturation (95°C for 30 s), annealing (Table 2), and extension (72°C for 30 s), with a final extension step of 10 min at 72°C. Amplicons were visualized on a 1.5% agarose gel under UV light to confirm the presence of target genes.

Table 2. Primers sequences, expected amplicon size, and annealing temperature for the target ARGs and 16 s rRNA genes

Gene Target	Antibiotic	Primer Sequence (5'→3')	Size (bp)	Annealing Temp (°C)	References
OXA-1	β-lactam	**F**: TAT CTA CAG CAG CGC CAG TG **R**: CGC ATC AAA TGC CAT AAG TG	199	60	[34]
bla_{NDM}	β-lactam	**F**: ATTAGCCGCTGCATTGAT **R**: CATGTCGAGATAGGAAGTG	154	55	[35]
bla_{TEM}	β-lactam	**F**: TTCCTGTTTTTGCTCACCCAG **R**: CTCAAGGATCTTACCGCTGTTG	113	60	[10]
16srRNA	Reference gene	**F**: CGGTGAATACGTTCYCGG **R**: GGWTACCTTGTTACGACTT	190	57	[36]

Quantitative PCR (qPCR) was subsequently conducted to quantify the abundance of the three target ARGs, using Maxima SYBR Green qPCR Master Mix 2X (ThermoFisher Scientific, USA) on a Bio-Rad CFX96 system (Bio-Rad Laboratories, Inc., Germany) [37]. Each qPCR reaction contained 10 µl of master mix, 0.2 µM of each primer, and 1 µl of DNA template (10 ng). Triplicate reactions were performed for each sample (biomass and effluent). At known concentrations, a calibration curve was constructed using purified PCR fragments of the target ARGs and the 16S rRNA gene (reference gene). Absolute quantification of ARGs was calculated based on the method described by Tichopad et al. [37], with the following formula: Xo = EAMP^(b-Cq), where Xo is the initial copy number, EAMP is the amplification efficiency (10^(-1/slope)), b is the y axis-intercept of the standard curve, and Cq is the PCR quantification cycle [38]. The fold change expressions were normalized using the endogenous reference gene 16S rRNA (ΔCt) and relative to the samples from AnMBR (biomass and effluent) before the addition of amoxicillin/flucloxacillin (ΔΔCt)

3. Results and Discussion

3.1. Organic Removal During Start-up Period

Before introducing the amoxicillin and flucloxacillin mixture, the AnMBR system underwent a start-up period to establish stable operational conditions and achieve a baseline performance. During this phase, the AnMBR was fed synthetic wastewater without β-lactam antibiotics (amoxicillin/ flucloxacillin), and COD removal efficiency was measured.

During the start-up, the permeate flux and TMP data indicated that no irreversible fouling occurred during the AnMBR operation. The mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) concentrations remained relatively stable at 3.8±0.42 g/l and 2.3±0.36 g/l, respectively. The average total COD concentration in the AnMBR influent was 490 ± 32 mg/L. COD removal efficiency gradually increased, reaching a steady state of approximately 94% after 40 days of operation (Figure 2). COD concentration results fall within the range reported in the literature that, ranging from 200 mg/l to over 1000 mg/l [39]. COD removal above 90% suggests effective treatment performance and AnMBR system achieving a steady-state COD removal efficiency [2,40].

3.2. Organic Removal under Antibiotic Exposure

The AnMBR system demonstrated remarkable resilience and high performance in terms of COD removal efficiency, even in the presence of increasing concentrations of the amoxicillin and flucloxacillin mixture. Under consistent operating conditions, introducing different antibiotic concentrations (50, 100, and 250 µg/L) resulted in a transient decrease in COD removal efficiency. However, the system rapidly recovered, returning to its initial high-performance levels over time. This adaptability and resilience can be attributed to the extended solids retention time (SRT) of 300 days, allowing the microbial community within the AnMBR to acclimate and develop antibiotic resistance mechanisms. The average COD removal efficiency remained consistently high, averaging 91±4.5%, even with the addition of antibiotics, as depicted in Figure 3. These findings underscore the robust nature of the AnMBR technology and its ability to maintain stable and efficient operation despite the introduction of potentially inhibitory substances.

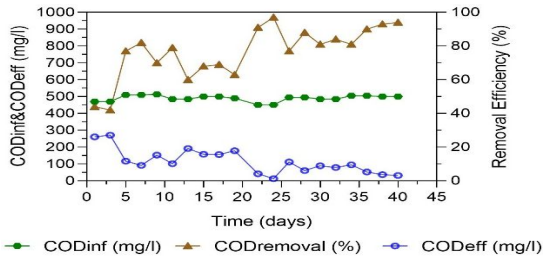

Fig. 2. COD removal efficiency over time during the AnMBR start-up period.

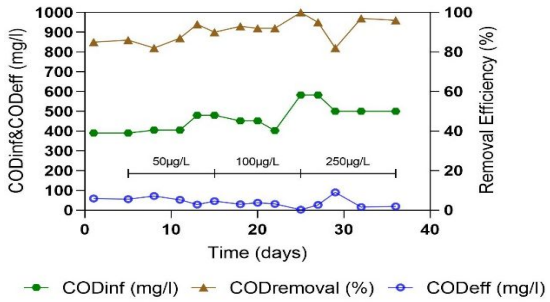

Fig. 3. COD removal efficiency over time in the AnMBR system under different antibiotic concentrations.

3.3. Biogas Production

Biogas production steadily increased during the start-up period, ultimately reaching a stable average daily production of 713 ± 150 mL/day. While fluctuations in production were observed, this is expected in lab-scale reactors where minor leakages and the use of biogas bags for measurement can introduce variability. The overall trend, however, demonstrates an increase in biogas production over time (Figure 4), even in the presence of antibiotics.

The average daily biogas production translates to approximately 142.5 L/g COD removed, considering the daily COD feeding of 5 g/d. This value is lower than the theoretical methane yield of 0.35 L CH_4/g COD. This discrepancy can be attributed to factors such as potential leakages, methane loss through the effluent, and the inherent limitations of lab-scale systems. Nevertheless, the observed biogas production aligns with values reported in the literature for AnMBRs treating various types of wastewater [3,39,40].

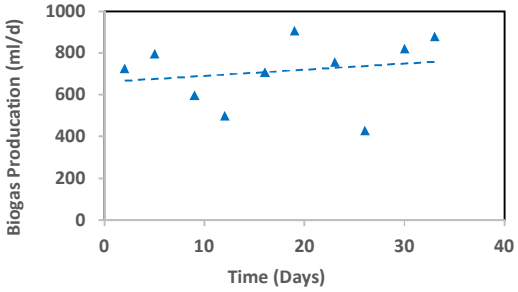

Fig. 4. Biogas production over time in the AnMBR system

3.4. ARGs Abundance and Diversity

3.4.1. ARGs Abundance in Effluent and Biomass

In order to evaluate the prevalence of β-lactamase resistance genes after supplementation of antibiotics to AnMBR system, the abundance values in the effluent of three target genes *oxa*-1, bla_{TEM}, and bla_{NDM} were quantified in both the AnMBR effluent and biomass using qPCR.

The results revealed that the gradual increase in the amoxicillin/flucloxacillin concentration in the AnMBR bioreactor led to an increase in the copy number of all tested antibiotic resistance genes in the effluent compared to control before the addition of antibiotic. The highest copy number was detected in *oxa*-1 at a concentration 250 µg/L (Figure 5a) followed by bla_{NDM} (Figure 5b) and bla_{TEM} (Figure 5c). While these genes were almost undetected in the AnMBR effluent during the startup phase, remarkable increases were observed after introducing antibiotics to the system. The high copy number of ARGs in the effluent are derived either from bacterial cell secretion when cells survive or through lysis when they die. These extracellular ARGs persist in the environment for a long period when adsorbed onto particulate substances such as clay or macromolecules [12,41]. It was reported that there is a positive correlation between the total relative abundance of ARGs and total concentrations of their related antibiotics; moreover, the multiple antibiotics promoted high spread rate of ARGs [7,42].

In contrast to the effluent, the biomass results showed a significant decrease in the abundance of all three ARGs at the higher antibiotic concentrations compared to the control phase (Figure 6). This observation suggests a potential shift in the microbial community composition within the biomass under increased antibiotic pressure. While the exact mechanisms underlying this decrease require further investigation, several factors could contribute to this trend. One possibility is that the higher antibiotic concentrations may inhibit the growth of certain bacterial populations carrying these ARGs, leading to their reduced abundance

in the biomass. Another possibility is that the increased antibiotic pressure may favor the growth of bacterial populations with alternative resistance mechanisms, potentially outcompeting those harboring the targeted β-lactamase genes. This observation aligns with previous studies that have reported varying responses of different ARG types to antibiotic exposure within AnMBR systems [4,33].

Analyzing the scale and magnitude of change in ARG abundance across the effluent and biomass (Figures 5 and 6) reveals further insights. It's important to note that the y-axis scales differ significantly between the effluent and biomass graphs, with the biomass generally exhibiting much higher ARG copy numbers overall. This is expected due to the membrane filtration in AnMBRs, which effectively removes bacteria and extracellular DNA carrying ARGs from the effluent. However, direct comparison of the magnitude of change between the effluent and biomass should be interpreted cautiously due to these scale differences.

Despite the varying scales, the contrasting trends in ARG abundance between the effluent (increasing) and biomass (decreasing) with increasing antibiotic concentrations highlight the complex dynamics of ARG propagation and removal within AnMBRs. The increase in ARG abundance in the effluent could be due to increased efflux or secretion of ARGs by bacteria under stress, or lysis of cells carrying those genes [41,43]. On the other hand, the decrease in the biomass might indicate that the bacteria carrying these specific ARGs are being outcompeted by others with different resistance mechanisms, or their growth is being inhibited at higher antibiotic concentrations.

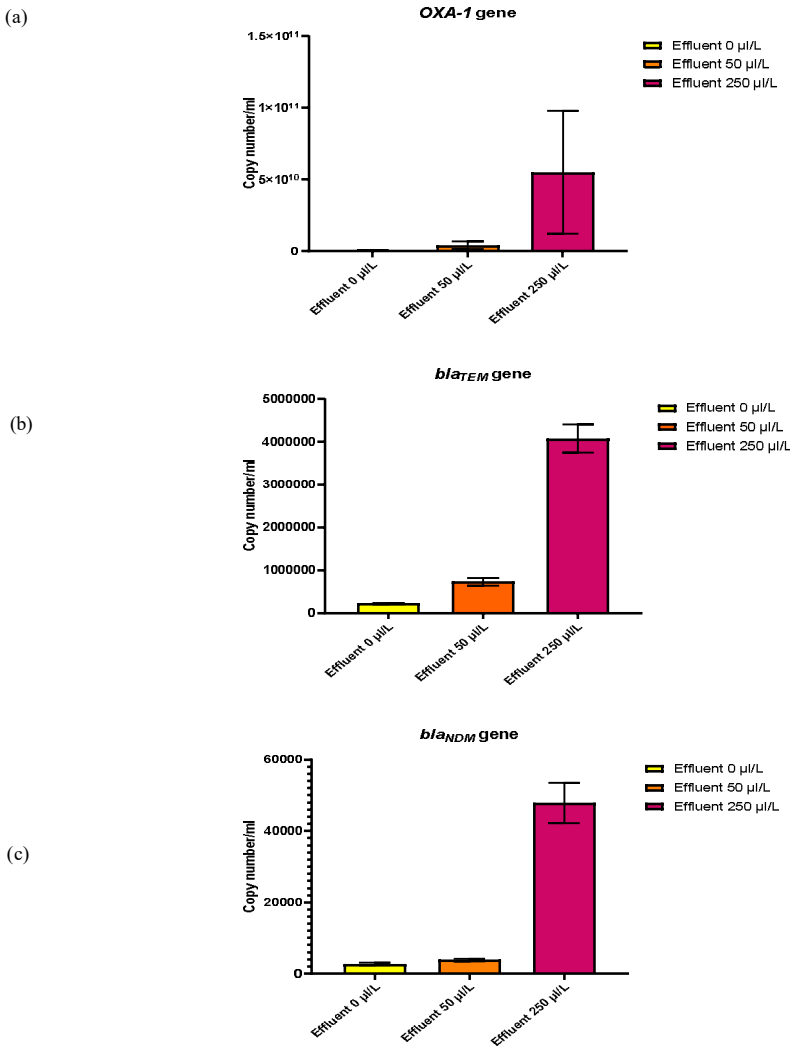

Fig. 5. Abundance of ARGs (copies/mL) in AnMBR effluent during the initial startup phase (no antibiotic) and subsequent exposure to antibiotic concentrations of 50 and 250 µg/L of amoxicillin/flucloxacillin.

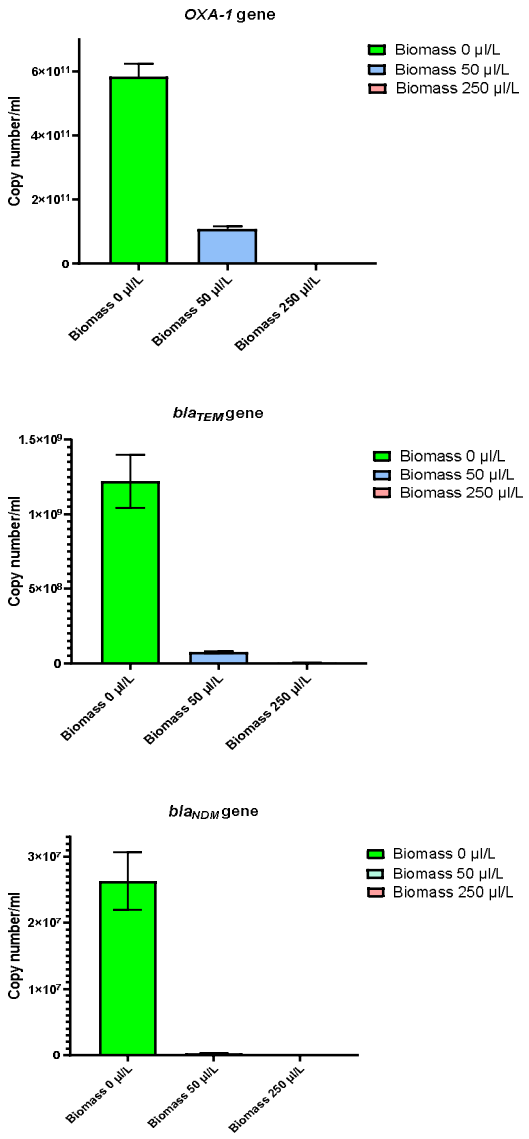

Fig. 6. Abundance of ARGs (copies/mL) in AnMBR biomass during the initial startup phase (no antibiotic) and subsequent exposure to antibiotic concentrations of 50 and 250 µg/L of amoxicillin/flucloxacillin.

These findings suggest that the microbial community within the AnMBR comprises diverse bacterial species with varying susceptibilities to the antibiotic mixture and different roles in ARG dynamics. As Zhang et al. [20] demonstrated, even under low selective pressure from a single antibiotic (tetracycline), shifts in bacterial communities and ARG profiles can occur in wastewater treatment systems. In our study, the presence of an antibiotic mixture likely exacerbates these shifts, leading to more complex interactions and outcomes. While some species may be negatively affected by the antibiotics, leading to a decrease in their abundance and the associated ARGs in the biomass, others may thrive under antibiotic pressure, potentially contributing to the increase in ARGs observed in the effluent. This highlights the importance of considering the complex interplay between different bacterial populations and resistance mechanisms when evaluating the impact of antibiotics on AnMBR performance and ARG dissemination [17,26].

3.4.2. ARGs Profiles

The fold change of ARGs in the AnMBR was also assessed (Figures 7 and 8). The results indicated that the presence of the amoxicillin and flucloxacillin mixture led to a shift in the three tested ARG, with certain genes becoming highly prevalent under

specific antibiotic concentrations. The highest fold change value in the effluent was recorded for the blaTEM gene, approximately 69.5 fold followed by *bla*$_{NDM}$ and *OXA-1* by 65.5 and 39.2 fold, respectively (Figure 7). Overall, ARG profiles of this investigation showed that, with increasing antibiotic concentrations (low or high concentration), the abundances of ARGs in the effluent increased. The ARGs propagation after antibiotic exposure was also reported in many studies in different wastewater treatment systems, that was explained by horizontal gene transfer under selective pressures [10,33,34].

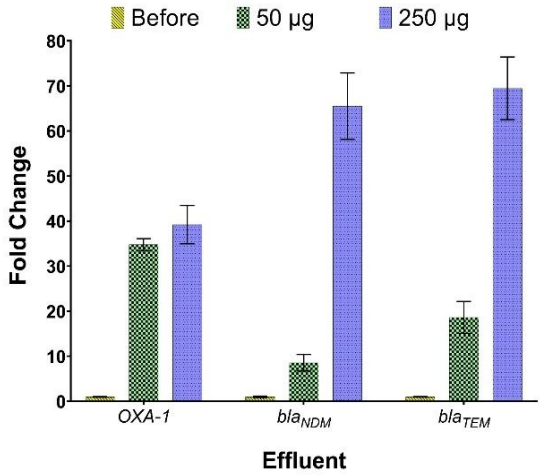

Fig. 7. The fold changes of oxa-1, blaTEM, and blaNDM genes detected in AnMBR effluent before (0 µg) and after the exposure of different concentration of amoxicillin/flucloxacillin (50, 250 µg). The fold changes expressions in each treatment were normalized using the endogenous reference gene 16S rRNA (ΔCt) and relative to the control samples from AnMBR effluent before the addition of antibiotics (ΔΔCt). The bars represent ΔΔCt mean ± SD from triplicate independent samples (n = 3).

However, in the biomass, the prevalence of antibiotics might follow very different directions, even those that confer resistance to the same antibiotic (Figure 8). It was observed that at the high concentration of antibiotics increased *bla*$_{TEM}$ concentration in the biomass in contrast to the other genes *bla*$_{NDM}$ and *OXA-1*. Indeed, the most frequently produced β-lactamases is the *bla*$_{TEM}$ genes in gram-negative bacteria [10,11]. This result was in line with previous study performed in a lab-scale AnMBR where *bla*$_{TEM}$ increased in the biomass but not the biofilms in contrast to other ARGs [9] suggesting that these genes might be induced in the effluent of AnMBR by entirely different groups of bacteria. Taken together, Plasmid mobility and conjugation, the presence of specific genes on plasmids, and the dynamics of microbial communities may all be connected to this phenomenon, even though its exact explanation is unknown [5,17,33].

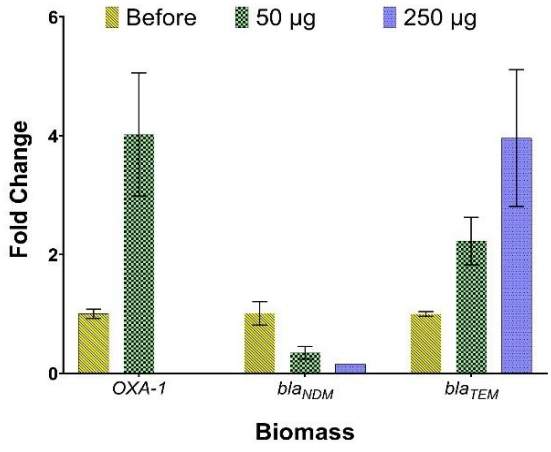

Fig. 8. The fold changes expressions from AnMBR biomass before and after the addition of amoxicillin/flucloxacillin. The samples were normalized using the endogenous reference gene 16S rRNA (ΔCt) and relative to the control (ΔΔCt) before the addition of antibiotic.

4. Conclusion

This study investigated the influence of an amoxicillin and flucloxacillin mixture on the performance of an AnMBR system. The results demonstrate the remarkable resilience and high performance of AnMBRs in treating municipal wastewater, even under continuous exposure to a mixture of commonly used antibiotics. The AnMBR maintained a stable and high COD removal efficiency (91 ± 4.5%) and consistent biogas production (713 ± 150 L/d) throughout the experiment, including phases with varying antibiotic concentrations. This highlights the system's robustness and potential for sustainable energy recovery, even in the presence of antibiotic contamination.

However, the observed shifts in ARG profiles within the biomass and effluent underscore the complex relationship between antibiotic exposure and the selection pressure for resistance genes. The increase in ARG abundance, particularly in the effluent, under antibiotic pressure emphasizes the need for continuous monitoring and optimization of AnMBR operation to minimize the potential for ARG dissemination and the subsequent spread of antibiotic resistance.

Future research should focus on elucidating the specific microbial community dynamics and operational strategies to enhance ARG removal efficiency in AnMBRs. This will ensure this technology's long-term sustainability and effectiveness in mitigating antibiotic resistance in wastewater and safeguarding public health.

Ultimately, this study contributes valuable insights into AnMBRs' response to antibiotic mixtures, highlighting the need for continued investigation into the complex interplay between wastewater treatment, antibiotic resistance, and environmental health. By further understanding and optimizing AnMBR performance under antibiotic pressure, we can move towards more sustainable and effective wastewater treatment practices that protect both human and environmental well-being.

5. Conflicts of interest

There are no conflicts to declare.

6. funding

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7. Acknowledgments

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8. References

[1] A. Charfi, N. Ben Amar, J. Harmand, Analysis of fouling mechanisms in anaerobic membrane bioreactors, Water Res. , 46 (2012) 2637–2650. doi:10.1016/j.watres.2012.02.021.

[2] K.K. Ng, X. Shi, H.Y. Ng, Evaluation of system performance and microbial communities of a bioaugmented anaerobic membrane bioreactor treating pharmaceutical wastewater, Water Res. , 81 (2015) 311–324. doi:10.1016/J.WATRES.2015.05.033.

[3] J. Ji, S. Sakuma, J. Ni, Y. Chen, Y. Hu, A. Ohtsu, R. Chen, H. Cheng, Y. Qin, T. Hojo, K. Kubota, Y.Y. Li, Application of two anaerobic membrane bioreactors with different pore size membranes for municipal wastewater treatment, Science of The Total Environment. , 745 (2020) 140903. doi:10.1016/J.SCITOTENV.2020.140903.

[4] A. Zarei-Baygi, M. Harb, P. Wang, L.B. Stadler, A.L. Smith, Microbial community and antibiotic resistance profiles of biomass and effluent are distinctly affected by antibiotic addition to an anaerobic membrane bioreactor, Environ Sci (Camb). , 6 (2020) 724–736. doi:10.1039/c9ew00913b.

[5] D. Cheng, H.H. Ngo, W. Guo, Y. Liu, S.W. Chang, D.D. Nguyen, L.D. Nghiem, J. Zhou, B. Ni, Anaerobic membrane bioreactors for antibiotic wastewater treatment: Performance and membrane fouling issues, Bioresour Technol. , 267 (2018) 714–724. doi:10.1016/J.BIORTECH.2018.07.133.

[6] S. Rodriguez-Mozaz, S. Chamorro, E. Marti, B. Huerta, M. Gros, A. Sànchez-Melsió, C.M. Borrego, D. Barceló, J.L. Balcázar, Occurrence of antibiotics and antibiotic resistance genes in hospital and urban wastewaters and their impact on the receiving river, Water Res. , 69 (2015) 234–242. doi:10.1016/j.watres.2014.11.021.

[7] H. Gao, F. Zhao, R. Li, S. Jin, H. Zhang, K. Zhang, S. Li, Q. Shu, G. Na, Occurrence and distribution of antibiotics and antibiotic resistance genes in water of Liaohe River Basin, China, J Environ Chem Eng. , 10 (2022) 108297. doi:10.1016/j.jece.2022.108297.

[8] B. Huang, H.C. Wang, D. Cui, B. Zhang, Z.B. Chen, A.J. Wang, Treatment of pharmaceutical wastewater containing B-lactams antibiotics by a pilot-scale anaerobic membrane bioreactor (AnMBR), Chemical Engineering Journal. , 341 (2018) 238–247. doi:10.1016/j.cej.2018.01.149.
[9] L. Ramadan, R. Deeb, C. Sawaya, C. El Khoury, M. Wazne, M. Harb, Anaerobic membrane bioreactor-based treatment of poultry slaughterhouse wastewater: Microbial community adaptation and antibiotic resistance gene profiles, Biochem Eng J. , 192 (2023) 108847. doi:10.1016/j.bej.2023.108847.
[10] D. Bibbal, V. Dupouy, J.P. Ferré, P.L. Toutain, O. Fayet, M.F. Prère, A. Bousquet-Mélou, Impact of three ampicillin dosage regimens on selection of ampicillin resistance in Enterobacteriaceae and excretion of blaTEM genes in swine feces, Appl Environ Microbiol. , 73 (2007) 4785–4790. doi:10.1128/AEM.00252-07.
[11] S.H. Mohamed, M.S. Khalil, M.I. Mabrouk, M.S.M. Mohamed, Prevalence of antibiotic resistance and biofilm formation in Klebsiella pneumoniae carrying fimbrial genes in Egypt, Res J Pharm Technol. , 13 (2020) 3051–3058. doi:10.5958/0974-360X.2020.00542.9.
[12] P. Ashwath, V.K. Deekshit, A. Rohit, I. Dhinakaran, I. Karunasagar, I. Karunasagar, D.S. Akhila, Biofilm Formation and Associated Gene Expression in Multidrug-Resistant Klebsiella pneumoniae Isolated from Clinical Specimens, Curr Microbiol. , 79 (2022) 73. doi:10.1007/s00284-022-02766-z.
[13] S. Aydin, B. Ince, O. Ince, Assessment of anaerobic bacterial diversity and its effects on anaerobic system stability and the occurrence of antibiotic resistance genes, Bioresour Technol. , 207 (2016) 332–338. doi:10.1016/j.biortech.2016.01.080.
[14] L. Yuan, Z.H. Li, M.Q. Zhang, W. Shao, Y.Y. Fan, G.P. Sheng, Mercury/silver resistance genes and their association with antibiotic resistance genes and microbial community in a municipal wastewater treatment plant, Science of the Total Environment. , 657 (2019) 1014–1022. doi:10.1016/j.scitotenv.2018.12.088.
[15] R.I. Aminov, R.I. Mackie, Evolution and ecology of antibiotic resistance genes, FEMS Microbiol Lett. , 271 (2007) 147–161. doi:10.1111/j.1574-6968.2007.00757.x.
[16] Z. Jian, L. Zeng, T. Xu, S. Sun, S. Yan, L. Yang, Y. Huang, J. Jia, T. Dou, Antibiotic resistance genes in bacteria: Occurrence, spread, and control, J Basic Microbiol. , 61 (2021) 1049–1070. doi:10.1002/jobm.202100201.
[17] A.H.A.M. Van Hoek, D. Mevius, B. Guerra, P. Mullany, A.P. Roberts, H.J.M. Aarts, Acquired antibiotic resistance genes: An overview, Front Microbiol. , 2 (2011) 1–27. doi:10.3389/fmicb.2011.00203.
[18] C.C.W. Young, D. Karmacharya, M. Bista, A.N. Sharma, T. Goldstein, J.A.K. Mazet, C.K. Johnson, Antibiotic resistance genes of public health importance in livestock and humans in an informal urban community in Nepal, Sci Rep. , 12 (2022) 1–13. doi:10.1038/s41598-022-14781-y.
[19] J. Davies, Origins and evolution of antibiotic resistance., Microbiologia. , 12 (1996) 9–16. doi:10.1128/mmbr.00016-10.
[20] Y. Zhang, M. Pei, B. Zhang, Y. He, Y. Zhong, Changes of antibiotic resistance genes and bacterial communities in the advanced biological wastewater treatment system under low selective pressure of tetracycline, Water Res. , 207 (2021) 117834. doi:10.1016/j.watres.2021.117834.
[21] W. Yan, R. Bai, S. Wang, X. Tian, Y. Li, S. Wang, F. Yang, Y. Xiao, X. Lu, F. Zhao, Antibiotic resistance genes are increased by combined exposure to sulfamethoxazole and naproxen but relieved by low-salinity, Environ Int. , 139 (2020) 105742. doi:10.1016/j.envint.2020.105742.
[22] A. Elawwad, A. Tarek, T.K. Abdelmoneim, S.M. Safwat, M.S.M. Mohamed, Prevalence of Antibiotic Resistance Genes (ARGs) in a small wastewater treatment plant in Egypt, World Congress on Civil, Structural, and Environmental Engineering. (2024). doi:10.11159/ICEPTP24.176.
[23] C.W. Knapp, J. Dolfing, P.A.I. Ehlert, D.W. Graham, Evidence of increasing antibiotic resistance gene abundances in archived soils since 1940, Environ Sci Technol. , 44 (2010) 580–587. doi:10.1021/es901221x.
[24] N. Fahrenfeld, K. Knowlton, L.A. Krometis, W.C. Hession, K. Xia, E. Lipscomb, K. Libuit, B.L. Green, A. Pruden, Effect of manure application on abundance of antibiotic resistance genes and their attenuation rates in soil: Field-scale mass balance approach, Environ Sci Technol. , 48 (2014) 2643–2650. doi:10.1021/es404988k.
[25] C.A. Quist-Jensen, F. Macedonio, E. Drioli, Membrane technology for water production in agriculture: Desalination and wastewater reuse, Desalination. , 364 (2015) 17–32. doi:10.1016/j.desal.2015.03.001.
[26] C. Verraes, S. Van Boxstael, E. Van Meervenne, E. Van Coillie, P. Butaye, B. Catry, M.A. de Schaetzen, X. Van Huffel, H. Imberechts, K. Dierick, G. Daube, C. Saegerman, J. De Block, J. Dewulf, L. Herman, Antimicrobial resistance in the food chain: A review, Int J Environ Res Public Health. , 10 (2013) 2643–2669. doi:10.3390/ijerph10072643.
[27] A. Zarei-Baygi, A.L. Smith, Intracellular versus extracellular antibiotic resistance genes in the environment: Prevalence, horizontal transfer, and mitigation strategies, Bioresour Technol. , 319 (2021) 124181. doi:10.1016/j.biortech.2020.124181.
[28] V.K. Sharma, N. Johnson, L. Cizmas, T.J. McDonald, H. Kim, A review of the influence of treatment strategies on antibiotic resistant bacteria and antibiotic resistance genes, Chemosphere. , 150 (2016) 702–714. doi:10.1016/j.chemosphere.2015.12.084.
[29] H. Sanderson, C. Fricker, R.S. Brown, A. Majury, S.N. Liss, Antibiotic resistance genes as an emerging environmental contaminant, Environmental Reviews. , 24 (2016) 205–218. doi:10.1139/er-2015-0069.
[30] J.R. Huddleston, Horizontal gene transfer in the human gastrointestinal tract: Potential spread of antibiotic resistance genes, Infect Drug Resist. , 7 (2014) 167–176. doi:10.2147/IDR.S48820.
[31] S. Mathur, R. Singh, Antibiotic resistance in food lactic acid bacteria—a review, Int J Food Microbiol. , 105 (2005) 281–295. doi:10.1016/J.IJFOODMICRO.2005.03.008.

[32] Tripathi, K.D. (2013) Essentials of Medical Pharmacology. 7th Edition, Jaypee Brothers Medical Publishers (P) Ltd., New Delhi, 816-835. - References - Scientific Research Publishing, (n.d.). https://www.scirp.org/reference/referencespapers?referenceid=1382183 (accessed October 12, 2024).
[33] A. Zarei-Baygi, M. Harb, P. Wang, L.B. Stadler, A.L. Smith, Evaluating Antibiotic Resistance Gene Correlations with Antibiotic Exposure Conditions in Anaerobic Membrane Bioreactors, Environ Sci Technol. , 53 (2019) 3599–3609. doi:10.1021/acs.est.9b00798.
[34] Y. Yang, T. Zhang, X.X. Zhang, D.W. Liang, M. Zhang, D.W. Gao, H.G. Zhu, Q.G. Huang, H.H.P. Fang, Quantification and characterization of β-lactam resistance genes in 15 sewage treatment plants from East Asia and North America, Appl Microbiol Biotechnol. , 95 (2012) 1351–1358. doi:10.1007/s00253-011-3810-5.
[35] D. Mantilla-Calderon, M.R. Jumat, T. Wang, P. Ganesan, N. Al-Jassim, P.-Y. Hong, Isolation and characterization of NDM-positive Escherichia coli from municipal wastewater in Jeddah, Saudi Arabia, Antimicrob Agents Chemother. , 60 (2016) 5223–5231.
[36] W.H. Gaze, L. Zhang, N.A. Abdouslam, P.M. Hawkey, L. Calvo-Bado, J. Royle, H. Brown, S. Davis, P. Kay, A.B.A. Boxall, Impacts of anthropogenic activity on the ecology of class 1 integrons and integron-associated genes in the environment, ISME J. , 5 (2011) 1253–1261.
[37] A. Tichopad, M. Dilger, G. Schwarz, M.W. Pfaffl, Standardized determination of real-time PCR efficiency from a single reaction set-up, Nucleic Acids Res. , 31 (2003). doi:10.1093/NAR/GNG122.
[38] G. Wei, T. Kehl, Q. Bao, A. Benner, J. Lei, M. Löchelt, The chromatin binding domain, including the QPQRYG motif, of feline foamy virus Gag is required for viral DNA integration and nuclear accumulation of Gag and the viral genome, Virology. , 524 (2018) 56–68. doi:10.1016/j.virol.2018.08.007.
[39] Z. Kong, J. Wu, C. Rong, T. Wang, L. Li, Z. Luo, J. Ji, T. Hanaoka, S. Sakemi, M. Ito, S. Kobayashi, M. Kobayashi, Y. Qin, Y.Y. Li, Large pilot-scale submerged anaerobic membrane bioreactor for the treatment of municipal wastewater and biogas production at 25 °C, Bioresour Technol. , 319 (2021). doi:10.1016/j.biortech.2020.124123.
[40] M. Galib, E. Elbeshbishy, R. Reid, A. Hussain, H.S. Lee, Energy-positive food wastewater treatment using an anaerobic membrane bioreactor (AnMBR), J Environ Manage. , 182 (2016) 477–485. doi:10.1016/j.jenvman.2016.07.098.
[41] K.M. Nielsen, P.J. Johnsen, D. Bensasson, D. Daffonchio, Release and persistence of extracellular DNA in the environment, Environ Biosafety Res. , 6 (2007) 37–53. doi:10.1051/ebr:2007031.
[42] P.J. Vikesland, A. Pruden, P.J.J. Alvarez, D. Aga, H. Bürgmann, X.D. Li, C.M. Manaia, I. Nambi, K. Wigginton, T. Zhang, Y.G. Zhu, Toward a Comprehensive Strategy to Mitigate Dissemination of Environmental Sources of Antibiotic Resistance, Environ Sci Technol. , 51 (2017) 13061–13069. doi:10.1021/acs.est.7b03623.
[43] L. Liu, X. Zou, Y. Gao, H. Li, Y. Cheng, X. Zhang, Q. Yuan, Differential dose-response patterns of intracellular and extracellular antibiotic resistance genes under sub-lethal antibiotic exposure, Ecotoxicol Environ Saf. , 260 (2023) 115070. doi:10.1016/j.ecoenv.2023.115070.