Water Science & Technology

IWA PUBLISHING

Water Science & Technology Vol 88 No 9, 2344 doi: 10.2166/wst.2023.324

The fate of sulfamethoxazole and trimethoprim in a micro-aerated anaerobic membrane bioreactor and the occurrence of antibiotic resistance in the permeate

Antonella Piaggio [a,*], Srilekha Mittapalli[b], David Calderón-Franco [c], David Weissbrodt [d], Jules van Lier [a], Merle de Kreuk [a] and Ralph Lindeboom [a]

[a] Faculty of Civil Engineering and Geosciences, Section Sanitary Engineering, Department of Water Management, Delft University of Technology, Stevinweg 1, 2628 CN, Delft, The Netherlands
[b] NX Filtration, Nanotechnology Research, Josink Esweg 44, 7545 PN, Enschede, The Netherlands
[c] Faculty of Applied Science, Department of Biotechnology, Delft University of Technology, Lorentzweg 1, 2628 CJ, Delft, The Netherlands
[d] Department of Biotechnology and Food Science, Norwegian University of Science and Technology, N-7491, Trondheim, Norway
*Corresponding author. E-mail: a.l.piaggio@tudelft.nl

AP, 0000-0003-4283-4495; DC-F, 0000-0002-1511-3839; DW, 0000-0002-6313-1652; JvL, 0000-0003-2607-5425; MdK, 0000-0002-5111-9461; RL, 0000-0002-0516-929X

ABSTRACT

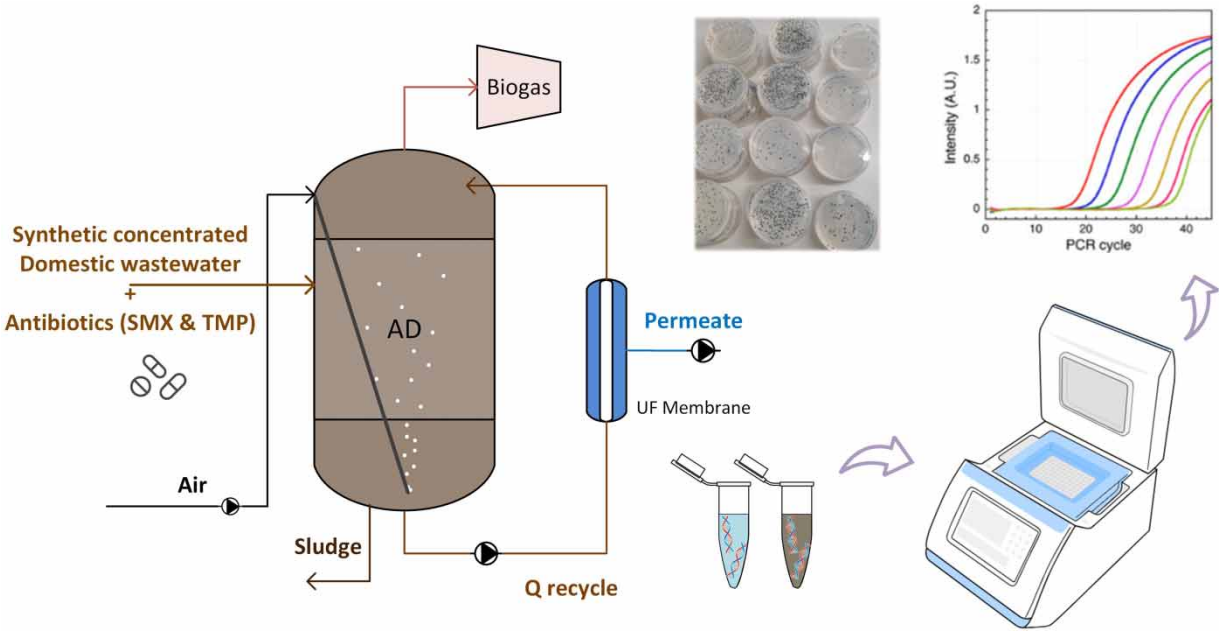
This study investigates the effects, conversions, and resistance induction, following the addition of 150 $\mu g \cdot L^{-1}$ of two antibiotics, sulfamethoxazole (SMX) and trimethoprim (TMP), in a laboratory-scale micro-aerated anaerobic membrane bioreactor (MA-AnMBR). TMP and SMX were removed at 97 and 86%, indicating that micro-aeration did not hamper their removal. These antibiotics only affected the pH and biogas composition of the process, with a significant change in pH from 7.8 to 7.5, and a decrease in biogas methane content from 84 to 78%. TMP was rapidly adsorbed onto the sludge and subsequently degraded during the long solids retention time of 27 days. SMX adsorption was minimal, but the applied hydraulic retention time of 2.6 days was sufficiently long to biodegrade SMX. The levels of three antibiotic-resistant genes (ARGs) (*sul1*, *sul2*, and *dfrA1*) and one mobile genetic element biomarker (*intI1*) were analyzed by qPCR. Additions of the antibiotics increased the relative abundances of all ARGs and *intI1* in the MA-AnMBR sludge, with the *sul2* gene folding 15 times after 310 days of operation. The MA-AnMBR was able to reduce the concentration of antibiotic-resistant bacteria (ARB) in the permeate by 3 log.

Key words: antibiotic resistance, antibiotics, micro-aerated AnMBR, SMX, TMP

HIGHLIGHTS

- Additions of SMX and TMP had a negligible effect on the MA-AnMBR performance.
- The laboratory-scale MA-AnMBR removed 86% of SMX and 97% of TMP.
- A 3-log removal of ARB was achieved between sludge and UF permeate.
- Relative abundances of ARGs were similar in sludge and permeate.
- TMP and SMX resistance is better assessed by the heterotrophic plate count of ARB.

GRAPHICAL ABSTRACT



ACRONYMS

AnMBR	anaerobic membrane bioreactor
ARB	antibiotic-resistant bacteria
ARG	antibiotic-resistant gene
COD	chemical oxygen demand
GC	gas chromatography
HGT	horizontal gene transfer
HRT	hydraulic retention time
K_{ow}	octanol–water partition coefficient
MA-AnMBR	micro-aerated anaerobic membrane bioreactor
MBR	membrane bioreactor
MGEs	mobile genetic elements
ORP	oxidation–reduction potential
qPCR	quantitative polymerase chain reaction
SMX	sulfamethoxazole
SRT	solids retention time
TMP	trimethoprim
TS	total solid
TSS	total suspended solid
UF	ultrafiltration
VFA	volatile fatty acid
VS	volatile solid
VSS	volatile suspended solid
WWTP	wastewater treatment plant

1. INTRODUCTION

Water demand has been increasing worldwide due to changes in consumption patterns, socioeconomic development, and population growth. Water consumption is expected to rise above one-quarter of the current consumption level by 2050 (WWAP 2019). The use of treated wastewater has risen as a possibility to alleviate water scarcity caused by water stress (Saidan *et al.* 2020). Nevertheless, to reclaim treated water, wastewater treatment plants (WWTPs) should be able to provide high-quality effluents. Most conventional WWTPs are not designed for the removal of antibiotics (Radjenovic *et al.* 2007;

Gros *et al.* 2010) and only minimal removal of pharmaceutical compounds can be observed in the primary treatment of wastewater (i.e., by coagulation, flocculation, and sedimentation) (Oulton *et al.* 2010).

Antibiotics are important components of human and veterinary medicines. Their consumption is increasing daily, leading to their occurrence in residual waters, such as municipal wastewater and urban and rural run-off. As much as 90% of the consumed antibiotics are excreted without any change in composition or functionality (Balakrishna *et al.* 2017). Among the available antibiotics, sulfamethoxazole (SMX, $C_{10}H_{11}N_3O_3S$) and trimethoprim (TMP, $C_{14}H_{18}N_4O_3$) are found in significantly high concentrations all over the world, as these are some of the most commonly used antibiotics in human and veterinary medications (van Boeckel *et al.* 2014). Sim *et al.* (2011) found high concentrations of SMX and TMP in WWTPs treating wastewater from the pharmaceutical industry, with maximum values reaching 309 and 162 $\mu g\,L^{-1}$, respectively.

Antibiotics can be removed or transformed by either biotic (biodegradation) or abiotic (sorption, ion exchange, complex formation with metal ions, and polar hydrophilic interactions) processes (Dı´az-Cruz *et al.* 2003; Michael *et al.* 2013). On the majority of WWTPs, the sorption and biodegradation of antibiotics occur in parallel. Pharmaceuticals can be biodegraded under aerobic, anoxic or anaerobic conditions, or in combination of all conditions, depending on the antibiotic. The centrally positioned amide group in SMX prevents its degradation under aerobic conditions. However, under anaerobic conditions it can be degraded by reductive cleavage of the molecule due to the adjacently located strong electron-withdrawing sulfonyl group. In the case of TMP, the substituted pyrimidine group can be readily biotransformed under anaerobic conditions (Alvarino *et al.* 2018). Figure 1 shows the chemical structure of SMX and TMP. Furthermore, the sorption potential of antibiotics is highly dependent on their molecular charge, polarity, and hydrophobicity, among other characteristics. Hydrophobic antibiotics have a great affinity to solid particles and therefore, have higher chances of being sorbed to sludge particles and reside long in the process to get degraded. The sorption capacity of antibiotics can be described as low, medium, or high, depending on their octanol-water partition coefficient (K_{OW}). High sorption is linked to log K_{OW} values above 4, while low sorption can be considered for antibiotics with log K_{OW} values below 0.25 (Rogers 1996). SMX and TMP properties are presented in Table 1.

The persistence of antibiotics in WWTPs and waterbodies can lead microbial communities to acquire antibiotic resistance. The O'Neill report (O'Neill 2014), commissioned by the United Kingdom government, predicts that by 2050, antibiotic-resistant infections will lead to 10 million annual deaths, with associated costs above 100 trillion USD. Furthermore, the World Health Organization established that the multi-resistance gained by bacteria is alarming and threatens global public health

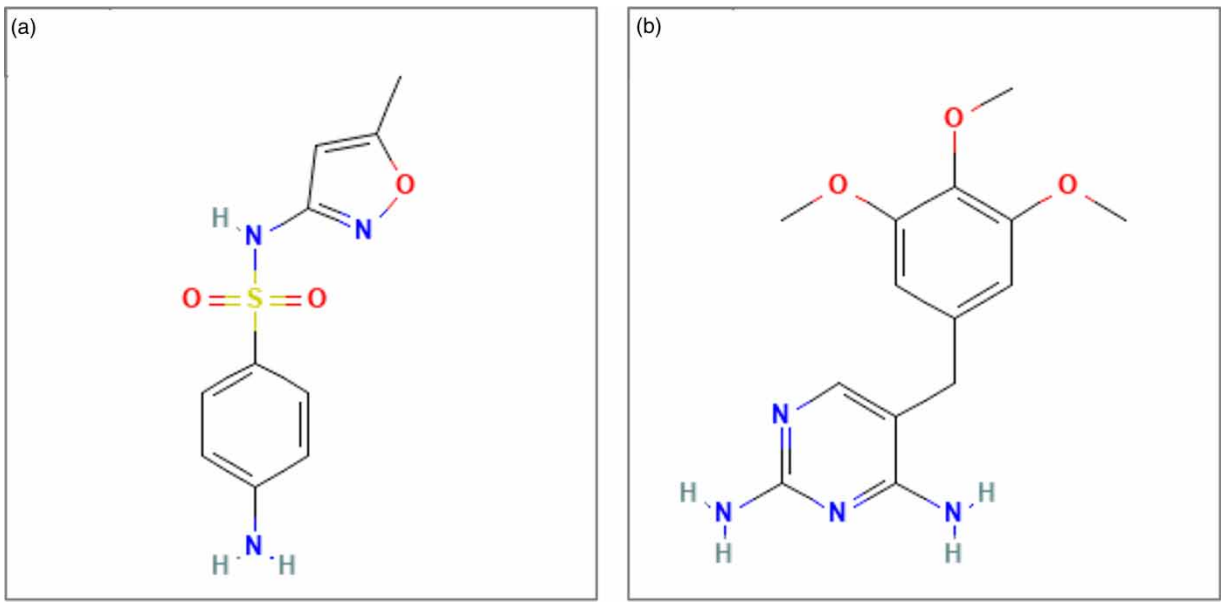


Figure 1 | Chemical structure of sulfamethoxazole (SMX) (a) and trimethoprim (TMP) (b) (N. C. f. B. I. NCBI CID 5329; N. C. f. B. I. NCBI CID 5578).

Table 1 | Selected antibiotics and their characteristics

Antibiotic chemical formula	Molecular weight (g.mol^{-1})	Charge[a]	Preferential removal pathway		log K_{ow}	Henry's constant (atm·m^3·mol^{-1})
Sulfamethoxazole (SMX) $C_{10}H_{11}N_3O_3S$	253.3	−	Anaerobic	Biodegraded	0.9	2.1×10^{-14}
Trimethoprim (TMP) $C_{14}H_{18}N_4O_3$	290.3	+	Anaerobic	Adsorbed	1.3	6.4×10^{-13}

Adapted from NCBI (N. C. f. B. I. NCBI US Government) and Alvarino *et al.* (2018). K_{ow} is the octanol–water partition coefficient and refers to the sorption capacity of antibiotics. The antibiotics removal pathways refer to the preferential ones, being either aerobic or anaerobic, and biodegraded or adsorbed.
Positive (+) or negative (−) charge is based on pH between 7 and 9.

(World Health Organization 2015). Wastewater catchment areas and WWTPs are considered one of the major points of antibiotic resistance release into the environment (Kümmerer 2009; Czekalski *et al.* 2012). Wastewater carries the complex cocktails of chemicals and biological contaminants released from anthropogenic activities. The high density of microbes in WWTPs has been frequently hypothesized to promote the transfer of antibiotic resistance via vertical and horizontal gene transfer in the presence of the antibiotics or other chemicals (Zarei-Baygi *et al.* 2019). The non-resistant bacteria can gain the resistance mechanisms from the antibiotic-resistant bacteria (ARB) via an exchange of mobile genetic elements (MGEs) like plasmids, integrons, and transposons, that contains antibiotic-resistant genes (ARGs) (Blair *et al.* 2015). Previous research indicated that the high solids retention times (SRTs) of WWTPs can result in an increased abundance of ARGs in conventional and membrane-based activated sludge systems (Xiao *et al.* 2017; Zhang *et al.* 2018).

Membrane bioreactors (MBRs) are potentially effective for treating pharmaceutical wastewater containing various antibiotics and other micropollutants (Oberoi *et al.* 2019). The long SRT and ultrafiltration (UF) membrane pore size are the main determining parameters for antibiotics removal in MBRs (Ji *et al.* 2020). Several authors found that the optimum SRT for enhanced antibiotics removal was around 30 days (Tadkaew *et al.* 2010; Xia *et al.* 2015; Nguyen *et al.* 2017). Due to its great affinity to solids, hydrophobic antibiotics can sorb to the sludge particles and then be subjected to biodegradation. The high SRT in MBRs promotes a diverse enzymatic activity due to the manifestation of slowly growing bacteria, which in parallel may support the degradation of the antibiotics (Göbel *et al.* 2007; Le-Minh *et al.* 2010).

Whether antibiotics are degraded via aerobic, anoxic or anaerobic conversion pathways, determines the need to apply a specific treatment technique, or treatments that combine both redox conditions.

In our previous work, we researched the feasibility of a laboratory-scale micro-aerated (MA) AnMBR, mimicking a full-scale digester equipped with a dissolved air flotation DAF unit for sludge retention instead of a membrane unit (Piaggio *et al.* 2023–2024). Results showed improved hydrolysis and negligible effects of the micro-aeration on operation and maintenance of the system. However, thus far, the removal of antibiotics and specifically SMX and TMP in an MA-AnMBR, remains unclear. Depending on the compound of concern, the application of micro-aeration in an AnMBR might impact their removal efficiency and rate. Since TMP and SMX removal is a mixture of bio-sorption and bio-conversion, the complete solids retention and high SRT provided by the MA-AnMBR system may enhance the degradation of both antibiotics. Furthermore, little is known about the effect of a membrane system on the growth and on the separation of ARB, as well as on the spreading of ARGs, in conjunction with the presence and removal of antibiotics in the wastewater.

Therefore, this study focused on the fate of SMX and TMP, in a laboratory-scale MA-AnMBR and their effect on the presence of antibiotic resistance in the MA-AnMBR permeate. Antibiotics removal mechanisms (adsorption and/or degradation) and the effects of adding SMX and TMP to the MA-AnMBR feed on its operation and performance were assessed. Measurements of ARGs and ARB from the sludge and permeate of the MA-AnMBR were performed to further understand the complexities and risks linked to the presence of antibiotics in domestic wastewater, and to address the efficiency of MA-AnMBRs to possibly contribute in reducing the spreading of antibiotic resistance from urban water systems.

2. METHODS

2.1. Experimental set-up

The laboratory-scale MA-AnMBR consisted a 6.5-L liquid volume continuous-flow stirred tank reactor equipped with an external inside-out tubular UF membrane, with a nominal pore size of 30 nm (Helyx, Pentair, Minnesota, United States).

Further membrane characteristics can be found in Supplementary material, Annex A. The set-up was equipped with feed, permeate, aeration, and recirculation pumps (Watson Marlow 120 and 323 U, Falmouth, United Kingdom). A Memosens CPS16D combined online sensor (Endress + Hauser, Reinach, Switzerland) was used to continuously measure the sludge oxidation–reduction potential (ORP), pH, and temperature. The MA-AnMBR operational conditions are based on the method described by Piaggio *et al.* 2023–2024, and are summarized in Table 2. Micro-aeration to the system was introduced in the reactors' bulk liquid, in three sets of 4 h of aeration followed by 4 h of no aeration. The total daily air volume introduced to the system was around 120 mL, which corresponds to 25 mL of O_2 (based on oxygen to air ratio of 0.21). The reactor setup and scheme are depicted in Figure 2. The concentrated influent consisted of synthetic wastewater, following an adapted recipe from Ozgun (Dereli *et al.* 2012). The synthetic feed had an average COD of 4.9 ± 0.6 g·L^{-1}, 66.4 ± 3.4 mgPO_4^{3+}-P·L^{-1}, and 244 ± 8 mgNH_4^+-N·L^{-1}. Feed composition and its recipe are presented in Supplementary material, Annex A.

2.2. Use of antibiotics SMX and TMP

Removal by adsorption and biodegradation of SMX and TMP was studied in both batch-scale systems and the continuously operated MA-AnMBR. From the literature, SMX and TMP concentrations in influent of WWTPs vary from 10 to 500 μg·L^{-1}. Considering that the synthetic influent of the MA-AnMBR is concentrated wastewater, 150 μg·L^{-1} of each antibiotic was added to the feed of the laboratory-scale MA-AnMBR. The addition of the antibiotics was done in steps and is described in the following. Moreover, SMX and TMP removal by adsorption was studied in batch tests (described in 2.5 hereafter) with concentrations between 10 and 150 μg·L^{-1}.

2.3. Reactor phases

The MA-AnMBR system was operated under stable conditions for 90 days, before the addition of antibiotics was started. Hereafter, this phase is referred to as *P.I*.

The subsequent phase called *P.II* refers to the time frame in which the two antibiotics, TMP and SMX, were added stepwise to the reactor feed, as follows. Both antibiotics were added in small concentration increments, to ensure that the microbial community was able to slowly adapt to the change in feed characteristics, without perishing. Firstly, TMP was added to the feed in three steps of increasing concentrations: 10, 50, and 150 μg·L^{-1}. The time lapse between each concentration shift corresponded to three hydraulic retention times (HRT), which was approximately one week. Thereafter, SMX addition started, and was done similarly to TMP in the same concentration steps and time. The whole *P.II* phase lasted 40 days.

Once both the SMX and TMP feed concentrations were 150 μg·L^{-1}, the reactor was continuously fed for a period of 120 days with the above-mentioned synthetic concentrated wastewater and 150 μg·L^{-1} antibiotics. The length of this period, of at least three SRTs (84 days), was selected to ensure that stable conditions were achieved. This phase is referred to as *P.III*.

Table 2 | Running conditions of a micro-aerated AnMBR

	Unit	Value
Feed flow	L·d^{-1}	2.5
Permeate flow	L·d^{-1}	2.3
Reactor volume	L	6.5
Temperature	°C	37
Hydraulic retention time (HRT)	d	2.6
Solids retention time (SRT)	d	28
Organic loading rate	gCOD·L^{-1}·d^{-1}	1.9
Recirculation flow	L·d^{-1}	1,300
Crossflow velocity	m·s^{-1}	0.6
Membrane flux	LMH	10.0

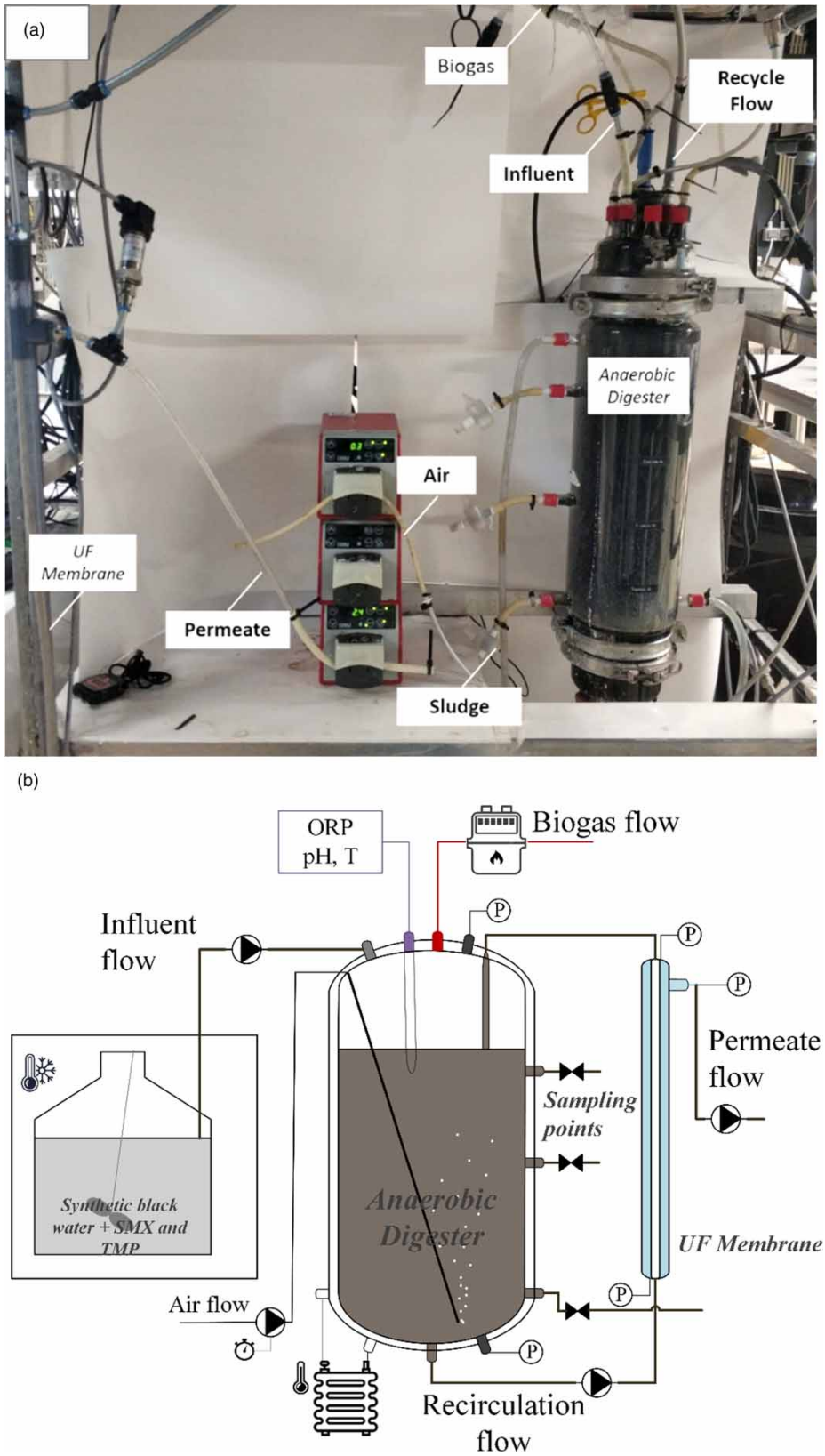


Figure 2 | Micro-aerated AnMBR setup (MA-AnMBR). Adapted from Piaggio *et al.* 2023–2024. (a) The MA-AnMBR laboratory-scale setup and (b) schematic representation of the laboratory-scale unit.

Finally, the influent without antibiotics was fed again to the system from day 250 onwards and monitored for a further 180 days, reaching a total operational time of 430 days. This last monitoring phase was denominated *P.IV*. A schematic overview of the reactor phases is displayed in Figure 3.

2.4. Analytical methods

COD measurements were conducted using HACH Lange test kits LCK 314, 514, and 014 (HACH, Tiel, The Netherlands). The nutrients, orthophosphate (PO_4^{3-}-P), total nitrogen (TN), ammonium-nitrogen (NH_4^+-N), and nitrate-nitrogen (NO_3^--N), were measured with HACH Lange test kits (LCK 238, 303, and 339, respectively). Total and volatile solids (in triplicates) were measured according to the APHA-Standard Methods (American Public Health Association, APHA 2005).

For the analysis of biogas, the gas samples were collected using 1.5-mL sterile syringes after which they were immediately injected into an Agilent tech 7890A gas chromatograph (GC) (Agilent, Santa Clara, CA, USA) equipped with a thermal conductivity detector. To analyze the composition of the gas samples, two separate columns, HP-PLOT U and a Molesieve GC column (Agilent 19095P-MS6, Santa Clara, CA, USA) of 60 m × 0.53 mm × 200 µm were used. Helium at a flow rate of 10 $mL{\cdot}min^{-1}$ was used as a carrier gas. The operational temperatures for the injector and detector were 40 and 200 °C, respectively (Ceron-Chafla *et al.* 2020).

The composition of the volatile fatty acids (VFA) of the MA-AnMBR sludge were measured using an Agilent tech 7890A GC, with helium as a carrier gas. The gas flow rate was 2.45 $mL{\cdot}min^{-1}$ (at 0.76 bar), and detector and injector temperatures were 225 and 240 °C, respectively. The liquid samples were collected in 2-mL Eppendorf every week and measured following the procedure described by García Rea *et al.* (2020). Acetic, caproic (IC6), and propionic acids are measured in $mg{\cdot}L^{-1}$, and the final VFA concentration is expressed in $mgCOD{\cdot}L^{-1}$.

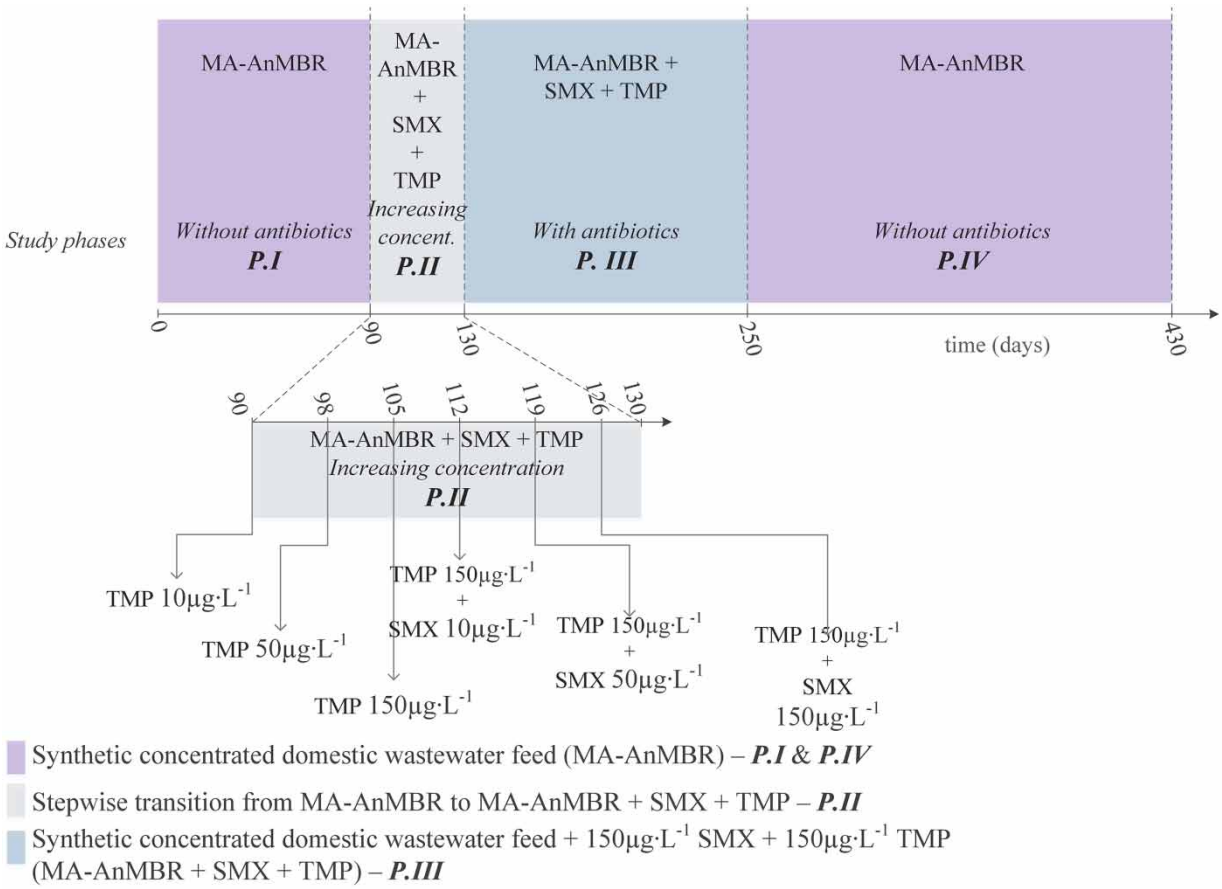


Figure 3 | Schematic representation of the reactor phases.

2.4.1. Antibiotics concentration measurement

Antibiotics concentrations in samples from batch experiments and the continuous-flow reactor were analyzed using liquid chromatography-tandem triple quadrupole mass spectrometry (LC-MS). For feed and permeate samples, before analysis, a volume of 2 mL of sample was centrifuged in a micro-centrifuge (Eppendorf, Hamburg, Germany) at 10,000 × *g* for a period of 3 min, and the supernatant was thereafter filtered through a 0.20 μm syringe filter (Chromafil® Xtra PES 20/25, Macherey-Nagel, Germany). Samples were stored at −20 °C for LC-MS analysis.

Antibiotic concentrations in the sludge from the continuous-flow reactor were measured using the methods described by Wijekoon *et al.* (2015). Homogenous sludge samples of 15 mL were centrifuged (Sorval ST 16R Centrifuge, Thermo Fisher Scientific, Waltham, MA, USA) at 14,000 × g for 15 min, and the supernatant was discarded. After freezing the remaining sludge samples at −80 °C for at least one day, the samples were freeze-dried for 24 h (BK-FD10, Biobase, Shandong, China). The dried sludge was then ground to a fine powder using a hand mortar and pestle, and a mass of 0.4 g was transferred to a tube, where 4 mL of methanol (>99%) was added. The samples were mixed with a vortex and sonicated for 10 min with an amplitude set up of 20% and temperature less than 60 °C (Branson 450 Digital Sonifier, Connecticut, USA). Afterwards, the sample was centrifuged at 3,300 × g for 15 min, and the supernatant was collected in a fresh 15 mL tube for further analysis. Finally, the solution was filtered through a 0.20 μm syringe filter (Chromafil® Xtra PES 20/25, Macherey-Nagel, Germany), and treated in a similar way to the permeate and liquid samples.

Chromatographic separation of the pharmaceuticals was performed by the ACQUITY UPLC® BEH C18 column (2.1 × 50 mm, 1.7 μm, Waters, Ireland) with a gradient elution of ultrapure water. Acetonitrile was the mobile phase and its flow rate was set to 0.35 mL.min^{-1} using an ACQUITY UPLC I-Class Plus pump (Waters, USA). Ultrapure water and acetonitrile (LC-MS grade, Biosolve, France) were acidified with 0.1% formic acid (LC-MS grade, Biosolve, France). Detection of the pharmaceuticals by mass spectrometry (Xevo TQ-S micro, Waters, USA) was conducted in the positive and negative electrospray ionization modes. The obtained data were analyzed and compared to internal standards, based on the methods and information described by Zheng *et al.* (2022).

2.4.2. Heterotrophic plate count

Microbiological screening and quantification were performed by spread plate method according to APHA-Standard Methods (American Public Health Association, APHA 2005). The total heterotrophic bacteria count was assessed by plating 0.1 mL of sample (either from the permeate or sludge) on a non-selective tryptone soya agar and low-nutrient Reasoner's 2A (R2A) agar. ARB were measured by adding concentrations of 50.4 mg·L^{-1} of SMX, or 16 mg·L^{-1} of TMP to the plate media (R2A). The antibiotics concentrations added to the R2A media were chosen based on the minimum inhibitory concentrations given by the Clinical and Laboratory Standards Institute (CLSI 2019) and studies performed by Zarei-Baygi *et al.* (2019, 2020).

2.5. Adsorption batch tests

Adsorption tests were performed in 250 mL glass bottles at 10 °C for a period of 7 h, to inhibit biodegradation. A volume of 100 mL of acclimated sludge from the first phase *P.I*, collected daily and stored at 4 °C, was used to perform all adsorption experiments. For SMX and TMP, three different antibiotic concentrations were tested: 10, 50, and 150 μg·L^{-1}. Each antibiotic concentration was added to the 250 mL glass bottles. All experiments were performed in triplicates, summing up to a total of 18 bottles and six different analysis conditions. Immediately after the addition of the antibiotics, the bottles were placed on the magnetic stirrer at 160 RPM (C-MAG HS7, IKA®, Staufen, Germany), for over 6 h. Sludge samples were collected in Eppendorf tubes of 2 mL volume and performed every 5 min for a period of 45 min, then every 15 min for 2 h, and finally, every half an hour for the next 4 h. After collecting, the samples were immediately centrifuged in a micro-centrifuge (Eppendorf, Hamburg, Germany), the supernatant was filtered through a 0.20 μm syringe filter, and the samples were stored at −20 °C until further analysis of the residual dissolved pharmaceuticals in the LC-MS.

2.6. Genetic analysis of ARGs

2.6.1. DNA extraction

Triplicate sludge and permeate samples from the MA-AnMBR were taken across the four studied phases *P.I to P.IV* for molecular analyses of their ARG content, after extraction of their total DNA. DNA extractions were performed following the protocol established by Albertsen *et al.* (2015), with minor adaptations. The extracted DNA was then used for quantitative polymerase chain reaction (qPCR) analysis of different concentrations of antibiotics resistant genes (see section 2.6.2).

While DNA could have also been used for microbial community analysis, this was not considered as part of the scope of this research. The use of a synthetic wastewater feed can lead to an approximation to what will occur in reality, but will not mimic exactly the conditions. Our previous publication (Piaggio *et al.* 2023–2024) contains further information on the microbial community shifts of the MA-AnMBR, when compared to a fully anaerobic system.

Homogenized sludge samples of 1.5 mL were transferred into an Eppendorf tube and centrifuged in a micro-centrifuge (Eppendorf, Hamburg, Germany). Approximately 50 mg of sludge pellets were added to DNA extraction tubes of the soil FastDNA spin kit (MP Biomedicals, Irvine, CA, USA).

MA-AnMBR permeate volumes of 2.0 L (per sample) of permeate were collected anaerobically and filtered through a 0.20 μm filter (Chromafil® Xtra PES 20/25, Macherey-Nagel, Germany). Afterwards, the filter was cut using scissors and the pieces were transferred in 1.5-mL Eppendorf tubes with tweezers (all used material was previously sterilized using autoclave (Fedegari FVA3/A1, Albuzzano, Italy)). Autoclave conditions were achieved by pressure-sterilization at 121 °C for 20 min.

The concentrations of the DNA extracts were measured using a Qubit dsDNA assay kit (Thermo Fisher, Waltham, MA, USA). Finally, the DNA samples were frozen at −25 °C until they were sent for gene amplification (Novogene Europe, Cambridge, United Kingdom).

2.6.2. Quantitative polymerase chain reaction (qPCR) analysis of selected ARGs

Three ARGs were selected for qPCR analysis on the DNA fractions extracted from the sludge and permeate of the continuous MA-AnMBR laboratory-scale system. The chosen ARGs targeted the sulfonamide resistance genes *sul1* and *sul2*, and dihydrofolate reductase gene *dfrA1*, to assess the potential resistance gained by the sludge by the addition of SMX and TMP, respectively. Aside from the ARGs, one mobile genetic element (MGE) biomarker was selected to investigate gene mobility, namely the class I integron-integrase gene *intI1* (Ma *et al.* 2017). The 16S rRNA gene was selected as a proxy to quantify total bacteria. Standards, primers, and mix solutions were based on the work performed by Calderón-Franco *et al.* (2021), and are given in Supplementary material, Annex B.

3. RESULTS

3.1. MA-AnMBR performance

Changes in the MA-AnMBR performance before and after adding the antibiotics are shown in Table 3. The COD removal was always above 97%, with permeate COD values that varied between 50 and 90 $mg{\cdot}L^{-1}$. The statistical difference between the reactor parameters was assessed using ANOVA single-factor between the MA-AnMBR parameters before and during the antibiotic's addition phases (*P.I* and *P.III*).

The reactor pH, system average biogas production, and biogas methane concentration showed statistical differences between the values obtained at the studied phases. The sludge pH decreased from 7.8 to 7.5 after the addition of antibiotics. Furthermore, while biogas production increased from 1.4 to 2.1, the change was not significant (p-value = 0.8) due to the higher standard deviation. The biogas methane content decreased, from 84 to 78%. While the CH_4 concentration decreased, the carbon dioxide biogas concentration doubled, from 7 to 16%. The increase in CO_2 concentration is aligned with the decrease in the system pH. No significant differences in sludge concentration, either suspended or total, were observed. Similarly, the nutrient content in the reactor permeate (as NH_4^+ and PO_4^{3-}) did not show statistically relevant changes after the addition of 150 $\mu g{\cdot}L^{-1}$ of SMX and TMP.

Antibiotics SMX and TMP removal was assessed during phase *P.II*, where the antibiotics were introduced stepwise until a concentration of 150 $\mu g{\cdot}L^{-1}$ each (during a period of 40 days in total), and during phase *P.III*, for 120 days. During *P.II*, TMP concentration in the MA-AnMBR permeate remained below 10 $\mu g{\cdot}L^{-1}$, and SMX values were below 20 $\mu g{\cdot}L^{-1}$. Once stable conditions were achieved at *P.III*, the SMX and TMP removal of the MA-AnMBR was 86 ± 5% and 97 ± 1%, respectively. Antibiotics concentrations adsorbed on the MA-AnMBR sludge were similar to the ones found in the permeate, 9 ± 4 $\mu g{\cdot}L^{-1}$ and 14 ± 6 $\mu g{\cdot}L^{-1}$ of TMP and SMX, respectively.

3.2. Antibiotics adsorption batch tests

Batch tests with MA-AnMBR sludge, taken from the reactor during *P.I*, were conducted to assess TMP and SMX adsorptions at 10 °C, for concentrations of 10, 50, and 150 $\mu g{\cdot}L^{-1}$.

Table 3 | Summary of the MA-AnMBR performance during the operational phases P.I., when the feed of the MA-AnMBR was synthetic concentrated wastewater (days 1–90), and P.III (days 130–250), when antibiotics were added to the synthetic feed

	Unit	*P.I.*	*P.III*	*p*-value
Chemical oxygen demand (COD) removal efficiency	%	99 ± 1	98 ± 1	0.3
Volatile fatty acids (VFA) accumulation	**mgCOD·L^{-1}**	**6.6 ± 1.1**	**10.8 ± 3.4**	**< 0.05**
Ortho-phosphate removal efficiency	%	59 ± 5	55 ± 7	0.4
Sulfate removal efficiency	%	>84[a]	>88[a]	0.8
Ammonium concentration in the permeate	mgNH_4^+-N·L^{-1}	694 ± 44	677 ± 49	0.1
Sludge total suspended Solids (TSS)	g·L^{-1}	6.1 ± 0.7	5.7 ± 0.1	0.9
Sludge volatile suspended Solids (VSS)	g·L^{-1}	3.3 ± 0.4	3.5 ± 0.1	0.7
Sludge total solids (TS)	g·L^{-1}	7.7 ± 1.3	7.5 ± 0.1	0.8
Sludge volatile solids (VS)	g·L^{-1}	3.7 ± 0.8	3.6 ± 0.2	0.9
Sludge pH	**–**	**7.8 ± 0.2**	**7.5 ± 0.1**	**< 0.05**
Sludge oxidation-reduction potential (ORP)	mV	−538 ± 14	−532 ± 12	0.2
Average biogas production	L·d^{-1}	1.4 ± 0.3	2.1 ± 0.6	0.8
Methane concentration in biogas	**%**	**84 ± 3**	**78 ± 6**	**0.05**
Carbon dioxide concentration in biogas	**%**	**7 ± 3**	**16 ± 2**	**< 0.05**

Values correspond to averages and standard deviations of samples (in triplicates). Values shown in bold correspond to those which had statistically relevant changes between the different reactor phases, based on a single-factor ANOVA.
[a]MA-AnMBR permeate had values of sulfate concentration below the detection limit. The removal was calculated based on the minimum detection value.

For all studied concentrations, the adsorbed TMP fraction was around 82% after 6 h, as shown in Figure 4. TMP had a high level of adsorption in the first 5 min of testing. TMP concentrations in the liquid after 5 min were below 30% of the initial concentrations applying 10 and 50 μg·L^{-1}, and 50% for the initial concentration of 107 μg·L^{-1}. A single-factor ANOVA statistical analysis was carried out to assess the adsorption differences between the different initial TMP concentrations (10, 50, and 150 μg·L^{-1}). No statistical differences were found after 6 h of testing (*p*-value of 0.2).

Adsorption of SMX onto the MA-AnMBR sludge was minimal, with values of 11% after 6 h of testing (Figure 4). No statistical differences were found (*p*-value of 0.4) between the observed adsorption at the different SMX concentrations.

3.3. Antibiotic-resistant bacteria

The levels of ARB in the MA-AnMBR bulk and permeate were measured by heterotrophic plate count in three out of the four study phases: *P.II*, *P.III*, and *P.IV*, while the total bacterial concentration was additionally measured during phase *P.I* (using the methods described in 2.4.2). Results are shown in Figure 5. Total bacteria retained in the MA-AnMBR system was in the order of 3 log in all studied phases, 99.9% (difference between the bacteria count in the reactor bulk and the UF permeate). Removal of ARB varied and depended on the experimental phase. No bacteria resistant to SMX or TMP were found in the permeate after 21 days of TMP supply (day 111; influent TMP concentration was 150 μg·L^{-1} and SMX was not yet added).

During *P.IV*, the MA-AnMBR influent was supplied again with a feed without antibiotics. No bacteria resistant to SMX nor TMP were detected anymore from the MA-AnMBR permeate. SMX-resistant bacteria followed a similar trend to TMP-resistant bacteria. SMX-resistant ones were removed by 5 log during *P.II* and by 2 log during *P.III*. Likewise, TMP-resistant bacteria were removed by 4 log during *P.II* and by 2 log during *P.III*. Furthermore, during *P.III*, the concentration of total bacteria in the permeate was similar to the ARB ones.

3.4. Antibiotic-resistant genes

The ARGs *sul1*, *sul2* and *dfrA1*, and the MGE *intI1* were measured during the four reactor phases from the MA-AnMBR sludge. All genes were already found in the sludge sample that was taken before the addition of the antibiotics, on day 90 of operation. During *P.II*, all four genes showed an increase in their relative abundance (per 16S rRNA gene), as shown in Figure 6. A peak in all four gene relative abundances was observed on day 112, which corresponded to the start of the addition of 10 μg·L^{-1} SMX.

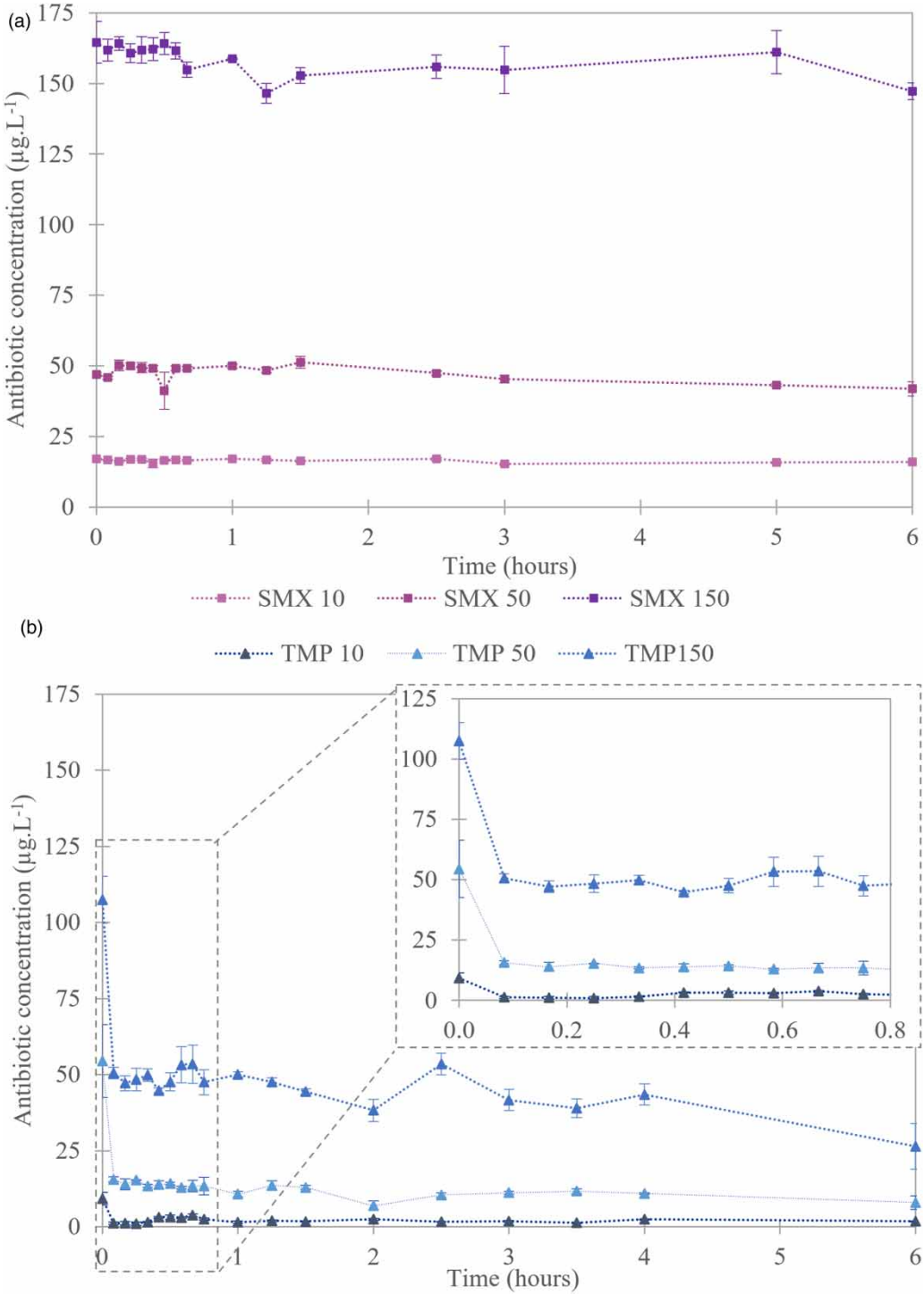


Figure 4 | Adsorption batch tests of antibiotics SMX (a) and TMP (b) at 10 °C, with MA-AnMBR sludge. The sludge total solid concentration was 4.1 and 3.9 $g{\cdot}L^{-1}$ for SMX (a) and TMP (b) samples, respectively. Additionally, a total volume of around 100 mL of sludge was used for each antibiotic concentration, and experiments were performed in triplicate.

Relative abundances of the *sul2* gene were highest among the analyzed genes, with an average difference corresponding to two orders of magnitude. Aside from the peak on day 112, *sul2* increased during *P.II* and *P.III* to a value of 3.2×10^{-1} gene copies (16S gene copies)$^{-1}$ at the end of *P.III* (day 249). During *P.IV*, the dosing of antibiotics in the influent was stopped,

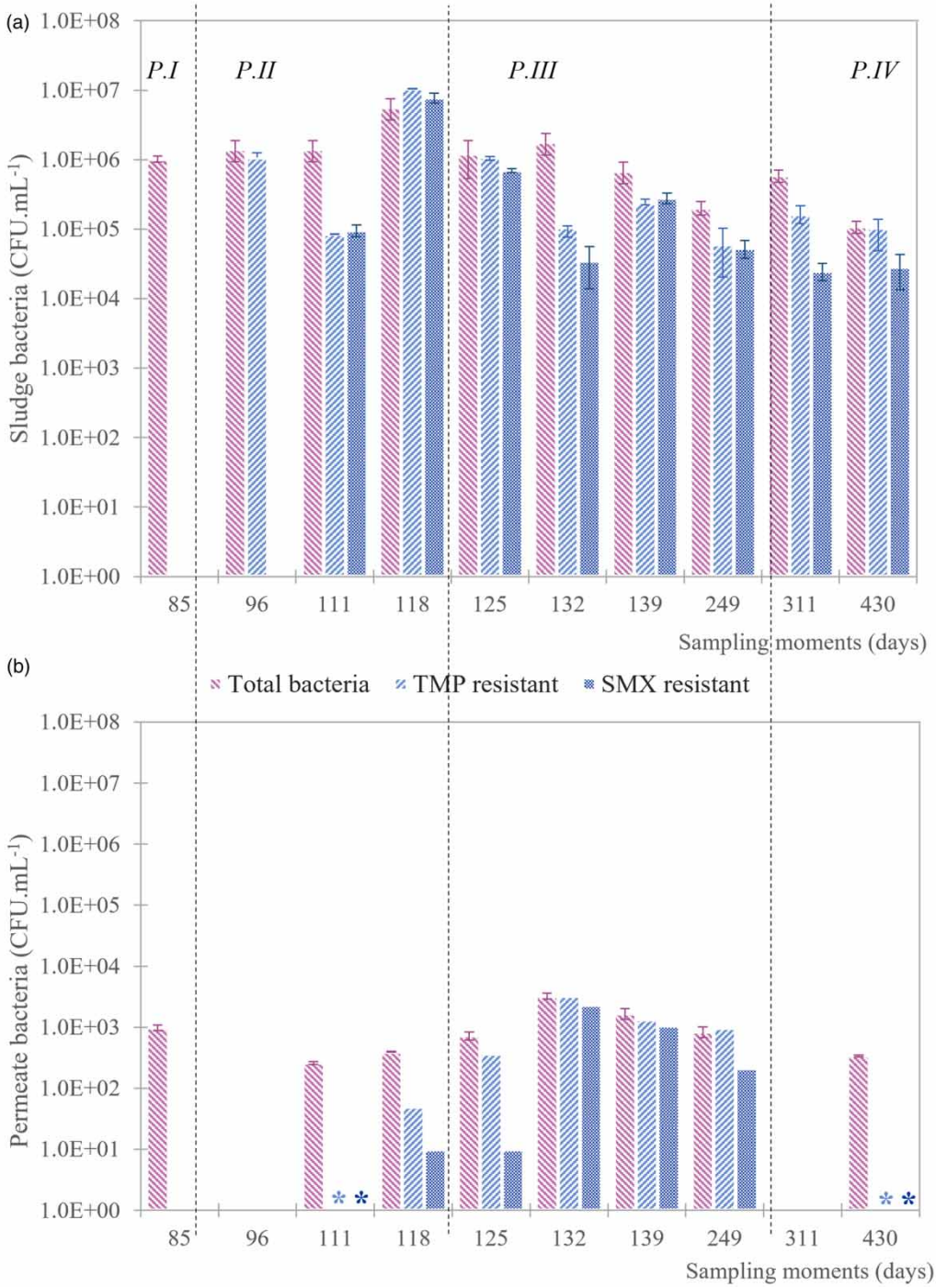


Figure 5 | Antibiotic-resistant and total bacteria in the MA-AnMBR: (a) shows the MA-AnMBR reactor bulk bacteria, (b) shows the MA-AnMBR UF permeate bacteria. The reactor phases are visualized with dotted vertical lines. P.I to P.IV refer to the different reactor phases. More information regarding the studied periods can be found in Figure 3. Days with * refer to measured samples with values below the detection limit. Blank days indicate no measurements.

however, after 62 days in *P.IV* (day 312), *sul2* reached even 4.1×10^{-1} gene copies (16S gene copies)$^{-1}$. This concentration decreased to only 2.4×10^{-1} gene copies (16S gene copies)$^{-1}$ on the last day of reactor operation (day 422). A reduction in gene copies in phase *P.IV* was also observed for *dfrA1* and *sul1*, indicating a loss of ARGs when antibiotics dosage to the

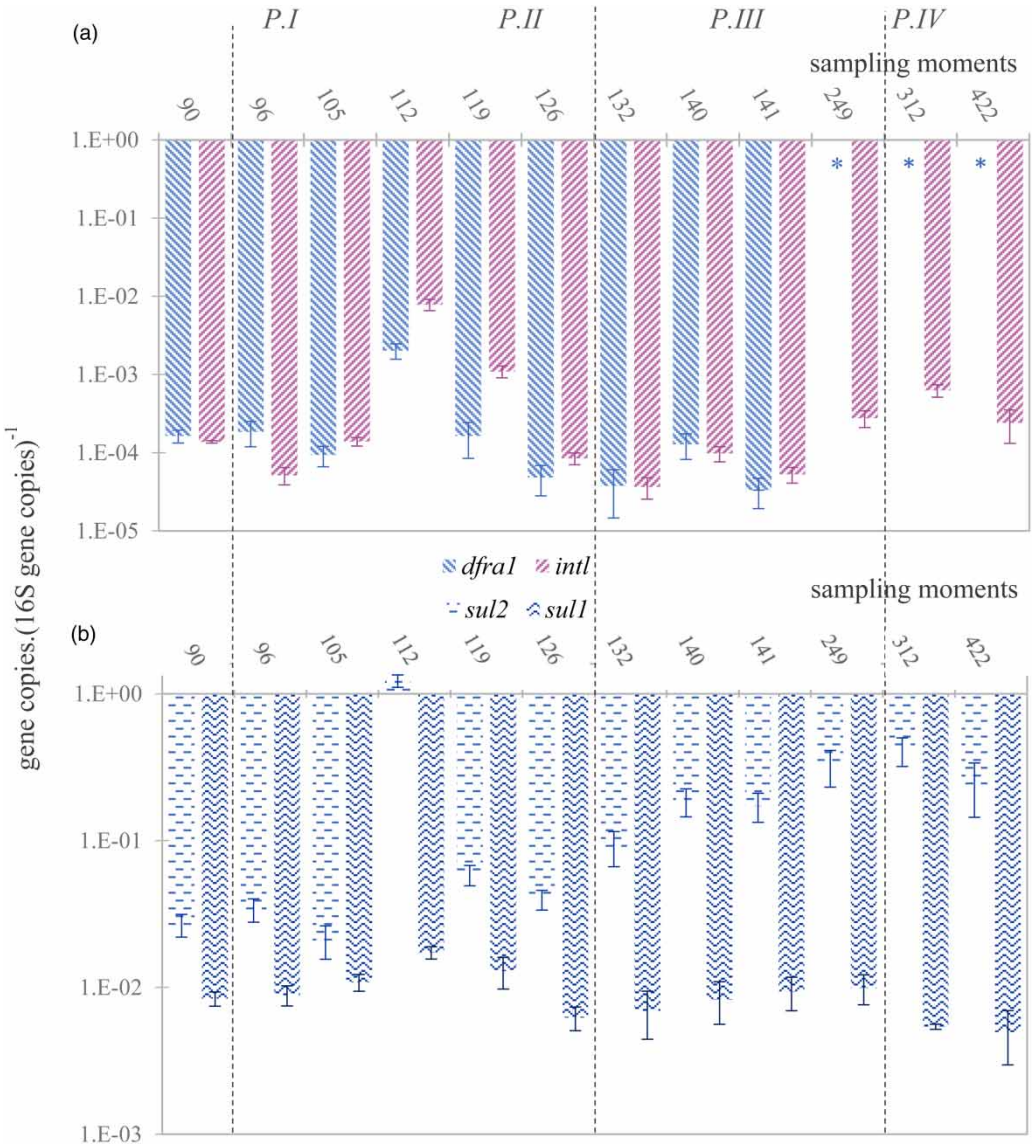


Figure 6 | Antibiotic-resistant gene copies per 16S gene copies of the MA-AnMBR sludge: (a) refers to dfrA1 and inlt1 genes concentration, (b) refers to sul1 and sul2 gene concentration. The reactor phases are visualized with dotted vertical lines P.I to P.IV refer to the different reactor phases. More information regarding the studied periods can be found in Figure 3. Values below the detection limit for dfrA1 (5.2E3 dfrA1 gene copies) are presented with *, and blank days indicate no measurements.

MA-AnMBR was stopped. Concentrations of *sul1* during *P.IV* were even found to be below the values measured before the start of the antibiotic's addition: 5.0×10^{-3} and 8.4×10^{-3} gene copies (16S gene copies)$^{-1}$ respectively. Finally, gene copies were below the detection limit of 5.2×10^{-3} for *dfrA1* at the end of *P.III* (249 days).

Gene copies from the UF permeate of the MA-AnMBR were measured during the first three reactor phases. UF permeate *dfrA1* gene copies were below the detection limit in all samples. For the rest of the studied genes, a difference of one to four orders of magnitude was found between the total abundance of gene copies (per mL of sample) in the bulk and the ones in the MA-AnMBR UF permeate, as shown for *sul1* in Figure 7. Apparently, the UF membrane of the MA-AnMBR retained the majority of the microorganisms that contained the studied genes, reducing 99.9% of the studied genes. Nevertheless, when considering the relative abundance of gene copies, the concentration of copies in the UF permeate and the reactor bulk tends to be in the same order of magnitude, as shown for the *sul1* gene in Figure 7. Permeate and reactor bulk concentrations of gene *sul2* and MGE *intI1* can be found in Supplementary material, Annex C.

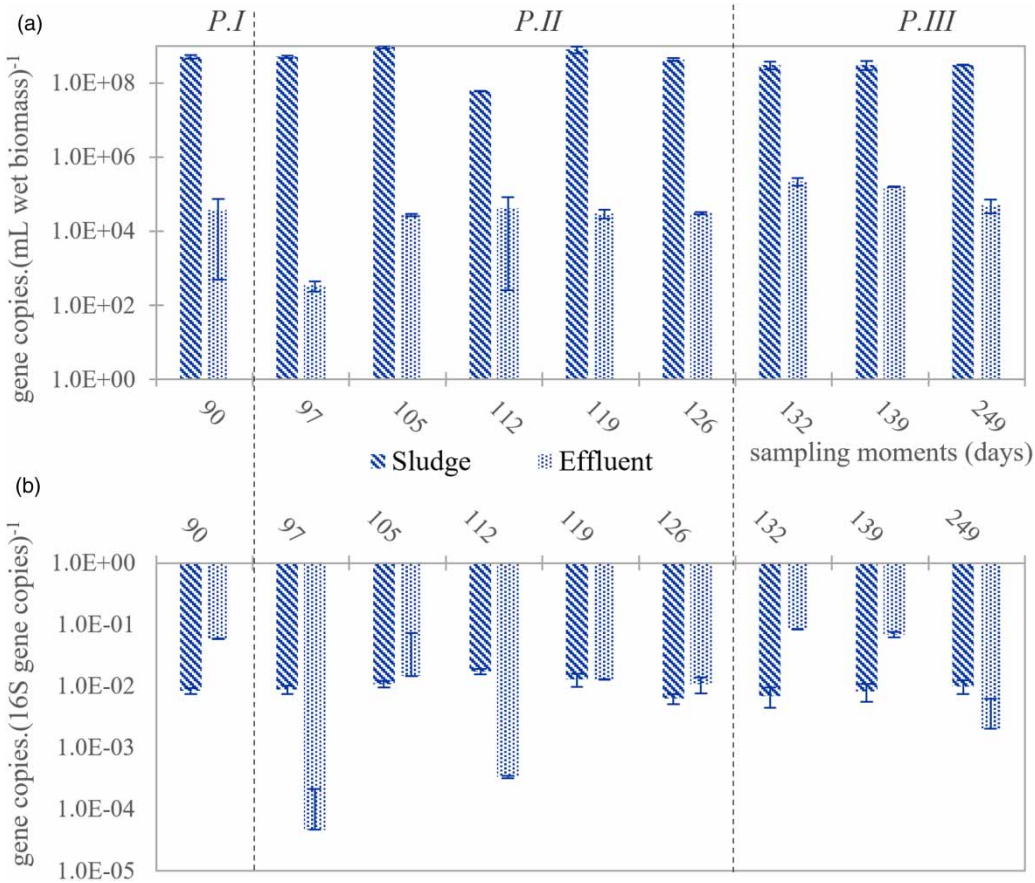


Figure 7 | The concentration of gene sul1 in the MA-AnMBR sludge and permeate: (a) shows the total gene copies per mL of wet biomass, while the values in (b) show the values of sul1 gene standardized per 16S gene copies. The reactor phases are visualized with dotted vertical lines P.I to P.IV refer to the different reactor phases. More information regarding the studied periods can be found in Figure 3.

4. DISCUSSION

4.1. Removal and consequences of TMP and SMX additions in the MA-AnMBR

Despite the addition of air in the MA-AnMBR, TMP removal was high, i.e., 97 ± 1%. A similar TMP removal of around 94% was obtained in a laboratory-scale AnMBR-treated synthetic sewage (Xiao *et al.* 2017). Feng *et al.* (2017) have found that TMP removal during pig manure digestion under strict anaerobic conditions exceeded 99% after 10 days of digestion. In contrast, a TMP removal of only 26.4% has been measured from conventional activated sludge processes that are mostly operated with aerobic conditions or in alternating redox conditions (Li & Zhang 2010). Previously, the changes in AnMBR performance with and without micro-aeration had been assessed: it was concluded that the given micro-aeration (reaching an oxygen over influent COD load of 1.0%) had a negligible impact on the reactor performance (Piaggio *et al.* 2023–2024). Thus, based on the efficiency measured in the MA-AnMBR, it can be inferred that the micro-aeration of the system has a negligible effect on TMP removal, and the MA-AnMBR is efficient in the removal of this antibiotic.

The log K_{ow} value of TMP and its positive charge at circumneutral pH results in quick adsorption onto the negatively charged biomass (Jia *et al.* 1996). Adsorption of TMP at 10 °C in batch tests showed a removal of TMP around 75% after 6 h of testing, as shown in Figure 4. In the continuous-flow MA-AnMBR, removal of TMP from the permeate reached 97%. From the results obtained in the adsorption tests, the sludge adsorption capacity for the highest TMP concentration tested (107 μg·L^{-1}) was around 200 μg·gTSS. The MA-AnMBR has a TSS content of 6 g·L^{-1} and total volume of 6.5 L, resulting in a total suspended biomass of 39 g. Considering the above-mentioned adsorption capacity, the MA-AnMBR is expected to be saturated with TMP after 21 days of operation. Hereafter, if no biodegradation of TMP would have occurred, TMP would have accumulated, reaching increased concentrations. Since the overall removal of TMP in the MA-AnMBR is

97%, it can be concluded that TMP was indeed degraded in the MA-AnMBR. Antibiotic concentration in the MA-AnMBR sludge was measured during *P.II* and *P.III*. The residual TMP concentration increased from *P.II* to *P.III* until it reached a plateau, with a concentration of 9 ± 4 μg·L^{-1}. Thus, TMP is quickly adsorbed onto the sludge and very likely subsequently digested. Apparently, the applied SRT of 27 days allowed enough time for the anaerobic degradation of TMP Alvarino *et al.* (2018), Feng *et al.* (2017).

The removal of SMX in the MA-AnMBR was 86 ± 5%. This value was in the same range, between 70 and 90%, of reported SMX removal in fully anaerobic laboratory-scale AnMBR units treating domestic wastewater (Zarei-Baygi *et al.* 2020; Harb *et al.* 2021; Oberoi *et al.* 2022). SMX removal in an aerobic activated sludge process has been reported to be much lower than in the MA-AnMBR, i.e., 39.1% (Li & Zhang 2010). ORP measurements showed that anaerobic conditions were kept in the MA-AnMBR even under micro-aeration, maintaining an ORP of −530 mV. While a high removal of SMX was obtained in the MA-AnMBR, batch adsorption tests at 10 °C showed SMX removal below 15% (Figure 4). The low adsorption of SMX onto the sludge is likely due to the negative charge of SMX at circumneutral pH, disfavoring its attraction to negatively charged biomass. The residual SMX concentration in the MA-AnMBR sludge was only 14 ± 6 μg·L^{-1} during *P.III*.

Most likely SMX was also degraded anaerobically in the MA-AnMBR, considering the measured removal efficiencies. With the measured low adsorption, the degradation rate of SMX is determined by the hydraulic loading rate, which resulted in an HRT of 2.6 days. The most important transformation reactions of the isoxazole ring of SMX and routes to co-metabolize SMX are hydroxylation, hydrogenation, acetylation, desulfurization, and reductive cleavage, among others (Jia *et al.* 2017; Tang *et al.* 2022). Some of the degradation pathways of SMX, like hydroxylation and desulfurization as part of acetylation, take less than 1 day, ensuring SMX degradation when HRTs < 1 day are applied (Tang *et al.* 2022). According to Dermer & Fuchs (2012), dehydrogenase enzymes play a key role in the hydroxylation of SMX under anaerobic conditions, and hydroxylation can be considered one of the main SMX degradation pathways. Results indicate that antibiotics like TMP, which easily adsorb to the sludge, are efficiently degraded in anaerobic reactors that are characterized by a long SRT. However, antibiotics that remain solubilized, like SMX, require a minimum HRT for efficient conversion. Regarding SMX, an HRT of 2.6 days was already sufficient for a high removal efficiency. For practical purposes, further research on optimizing HRT values is recommended.

While biogas quality changes from *P.I* to *P.III* were statistically significant, no change in biogas quantity was observed. Cetecioglu *et al.* (2015) have shown that only concentrations above 45 mg·L^{-1} of SMX were lethal to the microbial community and hence, inhibit biogas production. Zarei-Baygi *et al.* (2020) have concluded that after the addition of 250 μg·L^{-1} of SMX to a laboratory-scale AnMBR fed with synthetic wastewater, the abundance of methanogens remained the same, and the microbial community of biomass was stable throughout the study. Furthermore, Tang *et al.* (2022) concluded that addition of up to 2 mg·L^{-1} of SMX was beneficial for methane production in batch experiments, which was explained by the negative impact of SMX addition on acidogenic biomass, preventing the build-up of acid intermediates and low pH. As a consequence, the time required to reach the maximum methane production was distinctly shortened.

4.2. TMP and SMX ARB and ARGs abundance

The results of this long-term experiment indicated potential development of antibiotic resistance in the bacterial population when antibiotics are present in the wastewaters. In the MA-AnMBR permeate, no ARB bacteria were present in days 111 and 430. The latter sampling moment was performed when the MA-AnMBR was fed with synthetic feed without antibiotics, while the samples taken on day 111 corresponded to the moment when the MA-AnMBR was fed with the synthetic feed and 150 μg·L^{-1} of TMP. ARB measurements performed after day 111 showed an increase in both TMP and SMX relative concentrations (to the total heterotrophic bacterial count), as shown in Figure 5. The highest concentrations of TMP and SMX ARB in the UF permeate were obtained on *P.III*, having a relative abundance of 57 and 70%, respectively. Furthermore, TMP ARB in the MA-AnMBR bulk increased from 4% of the total heterotrophic bacteria count at *P.II* to 9% at *P.III* and finally 20% at *P.IV*. On the other hand, no significant difference was found in the relative abundance of the TMP and SMX ARB in the reactor bulk during the studied periods (p values of 0.3 and 0.8, respectively).

Both SMX- and TMP-resistant bacteria were measured in the MA-AnMBR permeate during phases *P.I*–*P.III*, and relative abundances of SMX- and TMP-resistant bacteria significantly increased during *P.II*, while the antibiotics were being added in steps (p-value below 0.05). Moreover, no resistant bacteria were found 180 days after the dosage of antibiotics to the MA-AnMBR was stopped (day 430, *P.IV*). While these results might indicate the loss of resistance once the antibiotics were removed from the feed solution, ARGs for SMX were still found in abundance in the MA-AnMBR permeate in *P.IV*,

on day 311, indicating the contrary. The ARG chosen for TMP, *dfrA1*, was below the detection limit in all phases of permeate samples. Thus, it is advised to assess the resistance toward this antibiotic in the MA-AnMBR on the ARB count, instead of the selected ARG.

The permeate of the laboratory-scale MA-AnMBR is rich in nutrients, such as nitrogen and phosphorus, and has an optimal temperature for bacterial re-growth. Furthermore, it is possible that some bacteria have passed the UF membrane of the MA-AnMBR. Lousada-Ferreira *et al.* (2016) have found that particles 100 times bigger than the nominal pore size were found in several membranes' permeates, even when membrane integrity was not compromised, leading to their conclusion that the nominal membrane pore size given by the manufacturer is rather an indication of the average membrane pore size but might be very different from the maximum values. The genes studied in this research had a length between 300 and 600 bp (Arabi *et al.* 2015), which using the rule of thumb that each base pair is about one third of a nm, entitles to lengths between 100 and 300 nm. Most municipal wastewater bacteria sizes vary between 1 and 100 μm (Levine *et al.* 1985). Therefore, it can be hypothesized that bacteria and genes might have passed the used membrane with nominal pore size of 30 nm. Nonetheless, the determined values around 10^3 CFU·mL^{-1} for the laboratory-scale MA-AnMBR permeate represented 3 log removal of total bacteria and ARB.

All measured ARGs and *intI1* relative abundances in the MA-AnMBR sludge and UF permeate increased during phase *P.II*, but only *sul1* and *sul2* in the sludge kept increasing during the phase *P.III*, as shown in Figure 6. These results are aligned with the results obtained by Guo *et al.* (2021), who found an increase in *intI1* mobile gene element, *sul1* and *sul2* due to an addition of oxygen equivalent to 1% of influent COD, in a sequencing batch reactor fed with blackwater. The increase in *sul1* and *sul2* genes due to the addition of SMX, has also been observed by Zarei-Baygi *et al.* (2019) and Blahna *et al.* (2006). While SMX resistance genes increased during *P.II* and *P.III*, no statistical differences were found in the relative concentration of SMX-resistant bacteria and total bacteria between these phases (*p*-value of 0.4). Furthermore, the relative abundance of ARG in the permeate was similar to the one obtained in the sludge. Thus, while the UF membrane can retain 99.9% of the measured bacteria, the bacteria present in the permeate has a similar abundance of ARGs.

Pearson correlation tests were conducted between the measured levels of SMX-resistant bacteria and TMP-resistant bacteria, and the relative abundances of the different genes in the samples taken from the reactor bulk and the permeate of the MA-AnMBR. A strong linear positive correlation was observed between TMP- and SMX-resistant bacteria in the reactor bulk and UF permeate, with ρ values of 0.83 and 0.97, respectively (Supplementary material, Annex D). Thus, SMX-resistant bacteria increased simultaneously with TMP-resistant bacteria, which might be attributed to the fact that both antibiotics were added in the same experimental phase to the MA-AnMBR, with only 20 days difference between the start of dosing TMP and SMX.

The gene *dfrA1* was positively correlated to all studied genes in the sludge samples of the MA-AnMBR. When a concentration of 10 μg·L^{-1} of SMX started to be added to the reactor on day 112, all studied genes reached their maximum concentration (Figure 6). Apparently, addition of SMX not only induced the concentration increase of *sul1* and *sul2*, i.e., the genes associated with this antibiotic, but also increased other ARGs. The change in *dfrA1* gene concentration could be linked to both SMX and/or TMP addition, and therefore, TMP resistance is not only linked to changes in *dfrA1* concentration. Results showed that plate counting of resistant bacteria is a better method of estimation for measuring resistance toward TMP than the relative abundance of the *dfrA1* gene.

Finally, all measured relative abundances of the *sul1*, *sul2*, *dfrA1*, and *intI1* genes decreased during phase *P.IV*, i.e., when the MA-AnMBR was fed without antibiotics. Thus, biological digestion in the MA-AnMBR is favorable for the reduction of ARG levels in the sludge. Several authors have stated that biological treatment and membrane systems are efficient for the removal of ARGs, especially *sul1* and *sul2* (Munir *et al.* 2011; Zarei-Baygi *et al.* 2019; Zheng *et al.* 2019). Nevertheless, considering the relative abundance of genes (vs. 16S rRNA gene copy number), the UF membrane system of the MA-AnMBR did not retain all microorganisms, and the permeate still had ARGs. Thus, while the biological treatment achieved an increased removal of ARGs, it was not efficient in retaining all microorganisms, and the UF permeate showed a similar ARGs relative abundance to the one in the reactor bulk. Thus, for the complete removal of ARB and microorganisms containing the studied ARGs, a subsequent treatment step should be implemented.

5. CONCLUSIONS

The fate of the SMX and TMP antibiotics was studied in a laboratory-scale MA-AnMBR fed with synthetic, concentrated domestic wastewater. The build-up of antibiotic resistance was assessed by measuring the concentrations of ARB, ARGs *sul1*,

sul2, *dfrA1*, and MGE *intI1* in the sludge and permeate. The effects of the additions of the antibiotics on the performance of the MA-AnMBR, their removal, and their relation to resistance induction were assessed for 430 days. The main conclusions of the research are the following:

- The addition of 150 $\mu g \cdot L^{-1}$ of SMX and TMP into the MA-AnMBR feed had negligible effects on the system performance. The sludge pH decreased from 7.8 to 7.5, which simultaneously entailed an increase in CO_2 concentration in the biogas (from 7 to 16%) and a decrease in the CH_4 partial pressure. These changes were statistically significant (p-value <0.05).
- A high removal of SMX and TMP was achieved in the laboratory-scale MA-AnMBR. SMX was poorly adsorbed into the sludge but rapidly degraded, reaching a total removal of 86%, measured in the MA-AnMBR permeate, relative to the influent. In contrast, TMP was rapidly adsorbed onto the MA-AnMBR sludge while the long SRT of the system guaranteed its degradation, achieving a total TMP removal of 97%. Thus, micro-aeration of the membrane system had no negative effects on the removal of the antibiotics.
- ARB and ARGs were found in both the MA-AnMBR sludge and permeate. No significant difference was found in the relative abundance of the TMP and SMX ARB in the reactor bulk during the studied periods. While the system was able to reduce the ARB concentration by 3 log, the relative ARG abundance was similar in the sludge and the UF permeate. The addition of SMX and TMP led to an increase in the relative abundance of all ARGs and *intI1* in the MA-AnMBR sludge.
- The relative abundance of the ARG *dfrA1* in the MA-AnMBR mixed liquor had a strong linear correlation with *sul1*, *sul2*, and the MGE *intI1*. However, changes in relative abundance of the genes were not linked to ARB. Thus, the gain of resistance to TMP or SMX is better assessed by the heterotrophic plate count of ARB than by molecular detection of the genes.
- The relative abundance of the MGE *intI1* in the sludge was positively and linearly correlated with all measured genes (ρ values above 0.82), reflecting the overall effect of antibiotics on the microbial gene pool.

ACKNOWLEDGEMENTS

This research was funded by NWO in the Netherlands (Project number 15424-2) and the Department of Biotechnology (DBT) in India (BT/IN/Indo-Dutch/19/TRS/2016), as part of the LOTUS-HR program (https://lotushr.org). Our gratitude goes to the lab technicians at the TU Delft Water Lab. Furthermore, we are thankful for the work done by Srilekha Mittapalli and Pravin Khande on the MA-AnMBR during their time at the TU Delft. The authors are grateful for Julia Gebert's contributions and supervision.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

REFERENCES

Albertsen, M., Karst, S. M., Ziegler, A. S., Kirkegaard, R. H. & Nielsen, P. H. 2015 Back to basics–the influence of DNA extraction and primer choice on phylogenetic analysis of activated sludge communities. *PloS one* **10** (7), e0132783. https://doi.org/10.1371/journal.pone.0132783.

Alvarino, T., Suarez, S., Lema, J. & Omil, F. 2018 Understanding the sorption and biotransformation of organic micropollutants in innovative biological wastewater treatment technologies. *Science of the Total Environment* **615**, 297–306. https://doi.org/10.1016/j.scitotenv.2017.09.278.

American Public Health Association, APHA 2005 *Standard Methods for the Examination of Water and Wastewater*, 21st edn. American Public Health Association, Washington, DC, USA, p. 1220, 2013.

Arabi, H., Pakzad, I., Nasrollahi, A., Hosainzadegan, H., Jalilian, F. A., Taherikalani, M., Samadi, N. & Sefidan, A. M. 2015 Sulfonamide resistance genes (sul) M in extended spectrum beta lactamase (ESBL) and non-ESBL producing Escherichia coli isolated from Iranian hospitals. *Jundishapur Journal of Microbiology* **8** (7). doi:10.5812/jjm.19961v2..

Balakrishna, K., Rath, A., Praveenkumarreddy, Y., Guruge, K. S. & Subedi, B. 2017 A review of the occurrence of pharmaceuticals and personal care products in Indian water bodies. *Ecotoxicology and Environmental Safety* **137**, 113–120. https://doi.org/10.1016/j.ecoenv.2016.11.014.

Blahna, M. T., Zalewski, C. A., Reuer, J., Kahlmeter, G., Foxman, B. & Marrs, C. F. 2006 The role of horizontal gene transfer in the spread of trimethoprim–sulfamethoxazole resistance among uropathogenic Escherichia coli in Europe and Canada. *Journal of Antimicrobial Chemotherapy* **57** (4), 666–672. https://doi.org/10.1093/jac/dkl020.

Blair, J., Webber, M. A., Baylay, A. J., Ogbolu, D. O. & Piddock, L. J. 2015 Molecular mechanisms of antibiotic resistance. *Nature Reviews Microbiology* **13** (1), 42–51. https://doi.org/10.1038/nrmicro3380.

Calderón-Franco, D., van Loosdrecht, M. C., Abeel, T. & Weissbrodt, D. G. 2021 Free-floating extracellular DNA: systematic profiling of mobile genetic elements and antibiotic resistance from wastewater. *Water Research* **189**, 116592. https://doi.org/10.1016/j.watres.2020.116592.

Ceron-Chafla, P., Kleerebezem, R., Rabaey, K., van Lier, J. B. & Lindeboom, R. E. 2020 Direct and indirect effects of increased CO2 partial pressure on the bioenergetics of syntrophic propionate and butyrate conversion. *Environmental Science & Technology* **54** (19), 12583–12592. https://doi.org/10.1021/acs.est.0c02022.

Cetecioglu, Z., Ince, B., Gros, M., Rodriguez-Mozaz, S., Barceló, D., Ince, O. & Orhon, D. 2015 Biodegradation and reversible inhibitory impact of sulfamethoxazole on the utilization of volatile fatty acids during anaerobic treatment of pharmaceutical industry wastewater. *Science of the Total Environment* **536**, 667–674. https://doi.org/10.1016/j.scitotenv.2015.07.139.

Czekalski, N., Berthold, T., Caucci, S., Egli, A. & Bürgmann, H. 2012 Increased levels of multiresistant bacteria and resistance genes after wastewater treatment and their dissemination into Lake Geneva, Switzerland. *Frontiers in Microbiology* **3**, 106. https://doi.org/10.3389/fmicb.2012.00106.

Dereli, R. K., Ersahin, M. E., Ozgun, H., Ozturk, I., Jeison, D., van der Zee, F. & van Lier, J. B. 2012 Potentials of anaerobic membrane bioreactors to overcome treatment limitations induced by industrial wastewaters. *Bioresource Technology* **122**, 160–170. https://doi.org/10.1016/j.biortech.2012.05.139.

Dermer, J. & Fuchs, G. 2012 Molybdoenzyme that catalyzes the anaerobic hydroxylation of a tertiary carbon atom in the side chain of cholesterol. *Journal of Biological Chemistry* **287** (44), 36905–36916. https://doi.org/10.1074/jbc.M112.407304.

Dı´az-Cruz, M. S., de Alda, M. a. J. L. & Barcelo, D. 2003 Environmental behavior and analysis of veterinary and human drugs in soils, sediments and sludge. *TrAC Trends in Analytical Chemistry* **22** (6), 340–351. https://doi.org/10.1016/S0165-9936(03)00603-4.

Feng, L., Casas, M. E., Ottosen, L. D. M., Møller, H. B. & Bester, K. 2017 Removal of antibiotics during the anaerobic digestion of pig manure. *Science of the Total Environment* **603**, 219–225. https://doi.org/10.1016/j.scitotenv.2017.05.280.

García Rea, V. S., Muñoz Sierra, J. D., Fonseca Aponte, L. M., Cerqueda-Garcia, D., Quchani, K. M., Spanjers, H. & Van Lier, J. B. 2020 Enhancing phenol conversion rates in saline anaerobic membrane bioreactor using acetate and butyrate as additional carbon and energy sources. *Frontiers in Microbiology* **11**, 604173. https://doi.org/10.3389/fmicb.2020.604173.

Göbel, A., McArdell, C. S., Joss, A., Siegrist, H. & Giger, W. 2007 Fate of sulfonamides, macrolides, and trimethoprim in different wastewater treatment technologies. *Science of the Total Environment* **372** (2–3), 361–371. https://doi.org/10.1016/j.scitotenv.2006.07.039.

Gros, M., Petrović, M., Ginebreda, A. & Barceló, D. 2010 Removal of pharmaceuticals during wastewater treatment and environmental risk assessment using hazard indexes. *Environment International* **36** (1), 15–26. https://doi.org/10.1016/j.envint.2009.09.002.

Guo, B., Yu, N., Weissbrodt, D. G. & Liu, Y. 2021 Effects of micro-aeration on microbial niches and antimicrobial resistances in blackwater anaerobic digesters. *Water Research* **196**, 117035. doi:10.1016/j.watres.2021.117035.

Harb, M., Zarei-Baygi, A., Wang, P., Sawaya, C. B., McCurry, D. L., Stadler, L. B. & Smith, A. L. 2021 Antibiotic transformation in an anaerobic membrane bioreactor linked to membrane biofilm microbial activity. *Environmental Research* **200**, 111456. https://doi.org/10.1016/j.envres.2021.111456.

Humphries, R., Bobenchik, A. M., Hindler, J. A. & Schuetz, A. N. 2021 CLSI. Overview of changes to the clinical and laboratory standards institute performance standards for antimicrobial susceptibility testing, M100. *Journal of clinical microbiology* **59** (12), 10.1128/jcm.00213-00221. https://doi.org/10.1128/jcm.00213-21.

Ji, J., Kakade, A., Yu, Z., Khan, A., Liu, P. & Li, X. 2020 Anaerobic membrane bioreactors for treatment of emerging contaminants: a review. *Journal of Environmental Management* **270**, 110913. https://doi.org/10.1016/j.jenvman.2020.110913.

Jia, X., Fang, H. H. & Furumai, H. 1996 Surface charge and extracellular polymer of sludge in the anaerobic degradation process. *Water Science and Technology* **34** (5–6), 309–316. https://doi.org/10.1016/0273-1223(96)00660-9.

Jia, Y., Khanal, S. K., Zhang, H., Chen, G.-H. & Lu, H. 2017 Sulfamethoxazole degradation in anaerobic sulfate-reducing bacteria sludge system. *Water Research* **119**, 12–20. https://doi.org/10.1016/j.watres.2017.04.040.

Kümmerer, K. 2009 Antibiotics in the aquatic environment–a review–part II. *Chemosphere* **75** (4), 435–441. https://doi.org/10.1016/j.chemosphere.2008.12.006.

Le-Minh, N., Khan, S., Drewes, J. & Stuetz, R. 2010 Fate of antibiotics during municipal water recycling treatment processes. *Water Research* **44** (15), 4295–4323. https://doi.org/10.1016/j.watres.2010.06.020.

Levine, A. D., Tchobanoglous, G. & Asano, T. 1985 Characterization of the size distribution of contaminants in wastewater: treatment and reuse implications. *Journal (Water Pollution Control Federation)* 805–816. Available from: https://www.jstor.org/stable/25042701.

Li, B. & Zhang, T. 2010 Biodegradation and adsorption of antibiotics in the activated sludge process. *Environmental Science & Technology* **44** (9), 3468–3473. https://doi.org/10.1021/es903490h.

Lousada-Ferreira, M., Van Lier, J. & Van Der Graaf, J. 2016 Particle counting as surrogate measurement of membrane integrity loss and assessment tool for particle growth and regrowth in the permeate of membrane bioreactors. *Separation and Purification Technology* **161**, 16–24. https://doi.org/10.1016/j.seppur.2016.01.033.

Ma, L., Li, A.-D., Yin, X.-L. & Zhang, T. 2017 The prevalence of integrons as the carrier of antibiotic resistance genes in natural and man-made environments. *Environmental Science & Technology* **51** (10), 5721–5728. https://doi.org/10.1021/acs.est.6b05887.

Michael, I., Rizzo, L., McArdell, C., Manaia, C., Merlin, C., Schwartz, T., Dagot, C. & Fatta-Kassinos, D. 2013 Urban wastewater treatment plants as hotspots for the release of antibiotics in the environment: a review. *Water Research* **47** (3), 957–995. https://doi.org/10.1016/j.watres.2012.11.027.

Munir, M., Wong, K. & Xagoraraki, I. 2011 Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. *Water Research* **45** (2), 681–693. https://doi.org/10.1016/j.watres.2010.08.033.

N. C. f. B. I. NCBI. *PubChem Compound Summary for CID 5329, Sulfamethoxazole*. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/ (accessed 4 September 2023).

N. C. f. B. I. NCBI. *PubChem Compound Summary for CID 5578, Trimethoprim*. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Trimethoprim (accessed 4 September 2023).

N. C. f. B. I. NCBI. *National Library of Medicine*. US Government. Available from: https://www.ncbi.nlm.nih.gov/ (accessed 3 March 2023).

Nguyen, T.-T., Bui, X.-T., Luu, V.-P., Nguyen, P.-D., Guo, W. & Ngo, H.-H. 2017 Removal of antibiotics in sponge membrane bioreactors treating hospital wastewater: comparison between hollow fiber and flat sheet membrane systems. *Bioresource Technology* **240**, 42–49. https://doi.org/10.1016/j.biortech.2017.02.118.

Piaggio, A. L., Sasidhar, K., Khande, P., Balakrishnan, M., van Lier, J. B., de Kreuk, M. K. & Lindeboom, R. E. F. 2023–2024 *Effects of low oxygen dosages on an anaerobic membrane bioreactor, simulating the oxygen load in an anaerobic digester-dissolved air flotation (AD-DAF) system.*

Oberoi, A. S., Jia, Y., Zhang, H., Khanal, S. K. & Lu, H. 2019 Insights into the fate and removal of antibiotics in engineered biological treatment systems: a critical review. *Environmental Science & Technology* **53** (13), 7234–7264. https://doi.org/10.1021/acs.est.9b01131.

Oberoi, A. S., Surendra, K., Wu, D., Lu, H., Wong, J. W. & Khanal, S. K. 2022 Anaerobic membrane bioreactors for pharmaceutical-laden wastewater treatment: a critical review. *Bioresource Technology* 127667. https://doi.org/10.1016/j.biortech.2022.127667.

O'Neill, J. 2014 'Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of Nations: December 2014,' Review on antimicrobial resistance.

Oulton, R. L., Kohn, T. & Cwiertny, D. M. 2010 Pharmaceuticals and personal care products in effluent matrices: a survey of transformation and removal during wastewater treatment and implications for wastewater management. *Journal of Environmental Monitoring* **12** (11), 1956–1978. https://doi.org/10.1039/C0EM00068J.

Radjenovic, J., Petrovic, M. & Barceló, D. 2007 Analysis of pharmaceuticals in wastewater and removal using a membrane bioreactor. *Analytical and Bioanalytical Chemistry* **387** (4), 1365–1377. https://doi.org/10.1007/s00216-006-0883-6.

Rogers, H. R. 1996 Sources, behaviour and fate of organic contaminants during sewage treatment and in sewage sludges. *Science of the Total Environment* **185** (1–3), 3–26. https://doi.org/10.1016/0048-9697(96)05039-5.

Saidan, M. N., Al-Addous, M., Al-Weshah, R. A., Obada, I., Alkasrawi, M. & Barbana, N. 2020 Wastewater reclamation in major Jordanian industries: a viable component of a circular economy. *Water* **12** (5), 1276. https://doi.org/10.1016/j.coesh.2018.03.008.

Sim, W.-J., Lee, J.-W., Lee, E.-S., Shin, S.-K., Hwang, S.-R. & Oh, J.-E. 2011 Occurrence and distribution of pharmaceuticals in wastewater from households, livestock farms, hospitals and pharmaceutical manufactures. *Chemosphere* **82** (2), 179–186. https://doi.org/10.1016/j.chemosphere.2010.10.026.

Tadkaew, N., Sivakumar, M., Khan, S. J., McDonald, J. A. & Nghiem, L. D. 2010 Effect of mixed liquor pH on the removal of trace organic contaminants in a membrane bioreactor. *Bioresource Technology* **101** (5), 1494–1500. https://doi.org/10.1016/j.biortech.2009.09.082.

Tang, T., Liu, M., Chen, Y., Du, Y., Feng, J. & Feng, H. 2022 Influence of sulfamethoxazole on anaerobic digestion: Methanogenesis, degradation mechanism and toxicity evolution. *Journal of Hazardous Materials* **431**, 128540. https://doi.org/10.1016/j.jhazmat.2022.128540.

van Boeckel, T. P., Gandra, S., Ashok, A., Caudron, Q., Grenfell, B. T., Levin, S. A. & Laxminarayan, R. 2014 Global antibiotic consumption 2000 to 2010: an analysis of national pharmaceutical sales data. *The Lancet Infectious Diseases* **14** (8), 742–750. https://doi.org/10.1016/S1473-3099(14)70780-7.

Wijekoon, K. C., McDonald, J. A., Khan, S. J., Hai, F. I., Price, W. E. & Nghiem, L. D. 2015 Development of a predictive framework to assess the removal of trace organic chemicals by anaerobic membrane bioreactor. *Bioresource Technology* **189**, 391–398. https://doi.org/10.1016/j.biortech.2015.04.034.

World Health Organization 2015 *Global Antimicrobial Resistance Surveillance System: Manual for Early Implementation*. World Health Organization, Geneva, Switzerland.

WWAP 2019 *The United Nations World Water Development Report 2019: Leaving No One Behind*. UNESCO, Paris, France.

Xia, Z., Xiao-Chun, W., Zhong-lin, C., Hao, X. & Qing-fang, Z. 2015 Microbial community structure and pharmaceuticals and personal care products removal in a membrane bioreactor seeded with aerobic granular sludge. *Applied Microbiology and Biotechnology* **99** (1), 425–433. https://doi.org/10.1007/s00253-014-5984-0.

Xiao, Y., Yaohari, H., De Araujo, C., Sze, C. C. & Stuckey, D. C. 2017 Removal of selected pharmaceuticals in an anaerobic membrane bioreactor (AnMBR) with/without powdered activated carbon (PAC). *Chemical Engineering Journal* **321**, 335–345. https://doi.org/10.1016/j.cej.2017.03.118.

Zarei-Baygi, A., Harb, M., Wang, P., Stadler, L. B. & Smith, A. L. 2019 Evaluating antibiotic resistance gene correlations with antibiotic exposure conditions in anaerobic membrane bioreactors. *Environmental Science & Technology* **53** (7), 3599–3609. https://doi.org/10.1021/acs.est.9b00798.

Zarei-Baygi, A., Harb, M., Wang, P., Stadler, L. B. & Smith, A. L. 2020 Microbial community and antibiotic resistance profiles of biomass and effluent are distinctly affected by antibiotic addition to an anaerobic membrane bioreactor. *Environmental Science: Water Research & Technology* **6** (3), 724–736. https://doi.org/10.1039/c9ew00913b.

Zhang, H., Jia, Y., Khanal, S. K., Lu, H., Fang, H. & Zhao, Q. 2018 Understanding the role of extracellular polymeric substances on ciprofloxacin adsorption in aerobic sludge, anaerobic sludge, and sulfate-reducing bacteria sludge systems. *Environmental Science & Technology* **52** (11), 6476–6486. https://doi.org/10.1021/acs.est.8b00568.

Zheng, W., Wen, X., Zhang, B. & Qiu, Y. 2019 Selective effect and elimination of antibiotics in membrane bioreactor of urban wastewater treatment plant. *Science of the Total Environment* **646**, 1293–1303. https://doi.org/10.1016/j.scitotenv.2018.07.400.

Zheng, X., Jiang, N., Zheng, H., Wu, Y. & Heijman, S. G. 2022 Predicting adsorption isotherms of organic micropollutants by high-silica zeolite mixtures. *Separation and Purification Technology* **282**, 120009. https://doi.org/10.1016/j.seppur.2021.120009.

First received 18 July 2023; accepted in revised form 23 September 2023. Available online 11 October 2023