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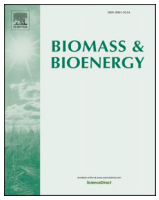

The effects of caffeine, gliclazide, and prazosin on the performance and microbial diversity in an up-flow anaerobic sludge blanket (UASB) reactor

Nur Alyaa Zahida Azizan [a], Hesam Kamyab [a,b,d,*], Ali Yuzir [c,**], Norhayati Abdullah [c], Irina Kirpichnikova [d], Bahareh Oryani [e], Shahabaldin Rezania [f]

[a] Malaysia-Japan International Institute of Technology (MJIIT), Universiti Teknologi Malaysia, Jalan Sultan Yahya Petra, 54100, Kuala Lumpur, Malaysia
[b] Department of Biomaterials, Saveetha Dental College and Hospital, Saveetha Institute of Medical and Technical Sciences, Chennai, 600 077, India
[c] Department of Chemical and Environmental Engineering (ChEE), Malaysia-Japan International Institute of Technology (MJIIT), Universiti Teknologi Malaysia, Jalan Sultan Yahya Petra, 54100, Kuala Lumpur, Malaysia
[d] Department of Electric Power Stations, Network and Supply Systems, South Ural State University (National Research University), 76 Prospekt Lenina, 454080, Chelyabinsk, Russian Federation
[e] Technology Management, Economics and Policy Program, College of Engineering, Seoul National University, 1 Gwanak-ro, Gwanak-gu, Seoul, 08826, Republic of Korea
[f] Department of Environment and Energy, Sejong University, Seoul, 05006, South Korea

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ABSTRACT

A laboratory-scale up-flow anaerobic sludge blanket (UASB) reactor was deployed in this study to examine the relationship between pharmaceutical compounds and anaerobic process performance. The reactor successfully biotransformed up to 87–99% of psychostimulant caffeine, anti-diabetic drug gliclazide, and anti-hypertensive drug prazosin during 92 days of operation. At the same time, fluctuations were recorded for the methane gas production, and also the domination of acetic acid and propionic acid in the presence of pharmaceutical compounds was measured. The results from 16s rRNA sequencing revealed that these compounds stimulated the growth of hydrogenotrophic methanogens, mainly *Methanobrevibacter* and *Methanobacterium*, while shifting the compositions of hydrolytic and fermentative bacteria. These outcomes proved the capability of the pharmaceutical compounds to influence the process performance by changing the microbial compositions in the anaerobic reactor.

1. Introduction

The occurrences of trace pharmaceutical compounds in Malaysian waterways have been linked to the incomplete removal of the compounds during treatment in conventional wastewater treatment processes [1]. Among the detected compounds were psychostimulant caffeine (CAF), anti-diabetic drug gliclazide (GCZ), and anti-hypertensive drug prazosin (PRZ), which range from as low as 6 ng L^{-1} up to more than 300 mg L^{-1} [2,3]. These compounds pose risks of bioaccumulation and toxicity to aquatic species [4,5] and have shown the prospect of changing the metabolic behaviour of microorganisms [6,7].

Anaerobic digestion (AD) has been acknowledged as one of the prospective enhancements to the existing conventional treatment processes to eliminate pharmaceutical compounds that are present in wastewater. The application of AD is favourable as it is known to be robust in treating various wastewater types, may operate with minimal energy requirements, and has biogas potential for energy recovery [8,9]. The application of AD to the process of treating pharmaceutical compounds revealed that most of the fractions undergo a biotransformation removal pathway and fewer residual fractions are generated in AD compared to the conventional aerobic processes [10].

The biotransformation of pharmaceutical compounds in AD may be achieved through direct or indirect co-metabolism of diverse microbial communities that grow under favourable reducing conditions in the process [11]. At the trace concentration levels, the removal of

* Corresponding author. Malaysia-Japan International Institute of Technology (MJIIT), Universiti Teknologi Malaysia, Jalan Sultan Yahya Petra, 54100, Kuala Lumpur, Malaysia.
** Corresponding author.
E-mail addresses: hesam_kamyab@yahoo.com, khesam2@live.utm.my (H. Kamyab), muhdaliyuzir@utm.my (A. Yuzir).

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pharmaceutical compounds is correlated with acidogenesis [12] and methanogenesis [13,14] based on the production of volatile fatty acids (VFAs) and methane gas. While the biotransformation process is reliant on microbial activities [15], previous findings have suggested that pharmaceutical compounds could, in return, trigger biological changes to the AD process.

Several AD studies [16–19] have reported extreme fermentation and inhibition of methanogenesis in the presence of antibiotics up to 50 mg L^{-1} concentration. These outcomes suggest that there is a mutual relationship between pharmaceutical compounds and the AD process stages. However, current findings are focused on antibiotics with antimicrobial characteristics [20,21]. Furthermore, the authors in Refs. [16–19] monitored the extreme effects of antibiotics at very high concentrations, which may not reflect the effect of other micropollutants that may also be prominent in general wastewater.

Conversely, the AD batch experiment conducted by Fáberová et al. [22] treating analgesics, beta-blockers, and psychostimulants resulted in varying stimulatory or inhibitory behaviour towards methane gas production. Their findings, however, reported the effects of individual compounds and did not provide information on the fermentation process. Furthermore, substrate limitation in the batch experiment may not have the same effect as continuous AD reactor operation.

To ensure the effectiveness of AD in treating pharmaceutical compounds, it is necessary to identify the effect of commonly-occurring pharmaceutical compounds on a continuous AD process. This study aims to assess the mutual relationship between pharmaceutical compounds and anaerobic process performance in an up-flow anaerobic sludge blanket (UASB) reactor. CAF, GCZ, and PRZ were introduced to an acclimatised UASB reactor and key performance indicators, including the removal efficiencies of the compounds, VFA concentration, and methane gas production, were monitored. Microbial diversity was also examined using the 16s rRNA sequencing method to correlate the effects of pharmaceutical compounds with changing anaerobic performance.

2. Materials and methods

2.1. Selection of pharmaceuticals

CAF, GCZ, and PRZ were selected for this study because of their significant presence in the Malaysian water environment [3], high consumption rate based on their therapeutic groups [23–25], toxicity effects [4–7], and different physicochemical characteristics. The details of these pharmaceuticals are provided in the Supplementary File.

Pure pharmaceutical standards (≥99% purity) of CAF, GCZ, and PRZ as prazosin hydrochloride were procured from Sigma Aldrich (USA). A mixed concentrated standard solution (1000 mg L^{-1}) was prepared by adding the standards into ultrapure water. The standards were further diluted in ultrapure water supplied by Thermo Scientific Smart2Pure (Sweden) to obtain the desired concentration for the experiments.

2.2. Synthetic wastewater

Glucose-enriched synthetic wastewater was derived as the feed for the start-up and experiment, as described by Azizan et al. [26]. Calcium chloride $CaCl_2$ (40 mg L^{-1}), magnesium sulfate $MgSO_4$ (40 mg L^{-1}), iron (II) sulfate $FeSO_4$ (32 mg L^{-1}), and potassium dihydrogen phosphate KH_2PO_4 (60 mg L^{-1}) were added as trace elements. All compositions were reagent grade procured from Merck (USA) except yeast extract (Difco, USA). The stock solution was prepared on a weekly basis and refrigerated at 4 °C when not in use. Subsequently, wastewater dilutions were made using tap water according to the desired COD concentrations during the start-up and the experiments.

2.3. UASB reactor set-up

The experiment was conducted using a single-stage stainless steel UASB bioreactor with a total working volume of 5 L. To maintain the process in a mesophilic condition (37 °C), hot water was circulated using a water circulator (Eyela, Japan) and flowed through the water jacket surrounding the reactor body. Wastewater flow was regulated using a peristaltic pump (Watson-Marlow, UK) connected to the inlet located at the bottom of the reactor. The gas outlet situated at the top of the reactor was connected through an optical bubble counter and a Tedlar gas bag (SKC, USA) for biogas collection. The conceptual diagram of the UASB bioreactor setup is provided in the Supplementary File.

2.4. Experimental protocol

The reactor was started up by inoculating the reactor with anaerobic digested sludge. The anaerobic digested sludge was obtained from a full-scale anaerobic digester tank located in Kuala Lumpur, Malaysia. For the start-up, the wastewater was flowed at an OLR of approximately 0.3 kg COD m^{-3} d^{-1} and increased step-wise at each steady state to 1.4 kg COD m^{-3} d^{-1} with HRT of 48 h. Sodium bicarbonate $NaHCO_3$ was added when required to maintain the reactor at a neutral pH range. Acclimatisation was reached on Day 149 with a final COD removal efficiency of 92%, methanisation of 48% of total biogas, and methane gas production of 0.147 L CH_4 d^{-1}. The UASB bioreactor performance throughout the start-up period is provided in the Supplementary File.

The experiment commenced by spiking the pharmaceutical compounds into the wastewater and continued for 92 d. The soluble COD of the wastewater was maintained at approximately 2500 mg L^{-1} and pH was regulated throughout the experiment. Each experimental condition was conducted consecutively upon reaching each steady state. The ratio of caffeine, gliclazide, and prazosin was maintained at 1:1:1 throughout the experiment. The details of the conditions are specified in Table 1. The concentration range was designed to account for the limitations of the pharmaceutical analysis while still allowing for the prediction of biotransformation under anaerobic conditions [27] and maintaining co-metabolism activities without jeopardising treatment stability. This range also corresponded to the occurrences of the compounds in the actual water environment [2,3] and the lower range of examined concentration in a continuous anaerobic reactor [28]. The bioreactor operated at HRT 36 for 48 h, considering the poor pharmaceutical removals by UASB at shorter HRT between 5.1 and 24 h [29,30].

2.5. Analytical method

2.5.1. Pharmaceutical compounds analysis

The analysis of pharmaceutical compounds was carried out on both wastewater effluent and sludge. Sampling for the effluent was carried out on a weekly basis and the sludge at the end of each experimental condition. Since the targeted compounds were non-volatile, the analysis was conducted using liquid chromatography instrumentation Waters ACQUITY UPLC-QDa (USA). All high performance liquid chromatography (HPLC) grade solvents used for the analysis were obtained from Merck (USA).

Each sample analyte of 30 μL was drawn by an autosampler and injected through a C18 column (2.7 μm 4.6 × 50 mm, Waters, Ireland). The temperature of the column was set to 40 °C. Mobile phases for the analysis were 0.1% formic acid in water (A) and acetonitrile (B) with a

Table 1
Experimental conditions during the UASB bioreactor operation.

Condition	Day	OLR (kg COD m^{-3} d^{-1})	HRT (h)	Concentration of pharmaceutical compounds (μg L^{-1})
I	150–164	1.59 ± 0.05	48	100
II	165–199	1.45 ± 0.13	48	400
III	200–220	1.82 ± 0.13	36	400
IV	221–241	1.84 ± 0.08	36	1000

flow rate of 0.8 mL min^{-1} throughout the elution. The elution began with B at 5% and increased linearly to 90% B for 4 min, then remained isocratic for 2 min. Next, the elution was returned to 5% B immediately and remained isocratic for 4 min. The separated compounds were detected using the QDa detector in the selective ion recording (SIR) positive mode with pre-set channels at 195, 324, and 384 *m/z* for CAF, GCZ, and PRZ, respectively. These channels were previously verified through injections of individual standards and quantification using the mass spectrometry (MS) scan mode (data are not disclosed in this paper).

All samples were pre-treated prior to the analysis. The effluent or aqueous samples were pre-treated using solid-phase extraction (SPE) according to a modified procedure [1]. SPE was carried out using a 20-sample vacuum manifold (Waters) and hydrophilic-lipophilic balance (HLB) cartridges (Oasis 60 mg 3 cc, Waters). First, all liquid samples were filtered twice using a 0.7 μm glass fibre filter (Whatman GF/F). The adsorbents in the cartridges were then preconditioned with 2 mL of methyl *tert*-butyl ether MTBE, 2 mL of methanol (MeOH) and 2 mL of ultrapure water (UPW). Under vacuum conditions, 100 mL of the liquid samples were loaded at a flow rate of 8 mL min^{-1}. The sorbents were then washed with 2 mL of UPW, and the cartridges were dried under a vacuum condition for 20 min. Next, elution was carried out by passing 5 x 1 mL of MTBE, 2 x 1 mL of acetone-MeOH (21:9, v/v) and 3 x 1 mL of acetone-MeOH (9:21, v/v), collected in a 12 mL glass tube. The mixed eluents were dried under a gentle stream of nitrogen gas until dryness, and reconstituted with 1 mL acetonitrile-UPW (5:95, v/v). The analytes were filtered with a 0.2 μm GHP syringe filter (Waters, USA) and transferred into borosilicate glass vials for the LC analysis.

The pretreatment of sludge or solid samples was done through ultrasonication extraction. UPW of 2.5 mL was added to 1.5 mL of solid samples and centrifuged at 3700 rpm (Thermo Scientific, USA) for 10 min. The liquid phase was then decanted, while the solid phase was added with 5 mL acetonitrile. The solid mixture was thoroughly mixed using a vortex mixer (LabServ, USA) and ultrasonicated for 20 min (Elmasonic S100, Germany). Next, the mixture was centrifuged again for 10 min at 3700 rpm. The supernatant was extracted and then filtered using a 0.2 μm GHP syringe filter and transferred into borosilicate glass vials for the LC analysis, as previously specified. The limits of quantification (LOQ), intra-day and inter-day relative standard deviation (RSD), and recoveries of the compounds in the aqueous and solid phases are specified in the Supplementary File.

2.5.2. Anaerobic process performance

The analyses of chemical oxygen demand (COD), VFAs, total suspended solids (TSS), and volatile suspended solids (VSS) were carried out in accordance with the Standard Methods of Examination of Water and Wastewater [31]. The COD analysis was facilitated by Hach High Range Plus COD digestion vials, while pH was measured using a pH meter (Ohaus, USA).

The VFAs analysis was conducted using ion chromatography instrumentation (ICS 5000+, Thermo Scientific, USA). All aqueous samples were pre-filtered using a 0.2 μm GHP syringe filter. Each sample volume of 4.5 mL was injected through a 4 mm × 250 mm analytical column (Dionex, IonPac™ ASII-HC). The targeted VFAs were eluted with eluent Dionex EGC III KOH and UPW as the carrier solution and detected using a conductivity detector (Dionex P/N 60–062433).

Biogas composition was determined using a gas chromatography-thermal conductivity detector (GC-TCD) Clarus® 690 GC (PerkinElmer, USA). An air-tight syringe was used to draw 5 mL of the biogas from the gas bag and taken for loop injection through a molecular sieve column (5 Å, 50 m, 0.53 mm, 50 μm, PerkinElmer, USA). The temperature of the column was set at 170 °C, while the detector was set to 200 °C. The nitrogen carrier gas flowed at a rate of 30 mL min^{-1}.

2.6. Molecular analyses

Sludge samples were harvested from the UASB bioreactor at the end of the start-up (Baseline), Condition II, and Condition IV. The sludge samples were brought to room temperature and immediately stored at −80 °C prior to molecular analyses. The analyses began with genomic DNA (gDNA) extraction of the UASB sludge, amplification of the target DNA through polymerase chain reaction (PCR), and next generation sequencing (NGS) for information on the microbial composition of each sample. All extraction and sequencing work was outsourced to an external laboratory.

Initially, the gDNA extraction was conducted using FastDNA™ Spin Soil Kit (MP Biomedicals) and confirmed using gel electrophoresis. Sample aliquots of 1 μL gDNA were run on a 1% TAE agarose gel at 100V for 60 min and compared against a positive control provided by a template containing 50 ng of bacterial gDNA. Meanwhile, the gDNA was quantified using the Implen NanoPhotometer® N60/N50 spectrophotometer. The gDNA was then subjected to an amplicon PCR quality control. A volume of 3 μL PCR product aliquots was run on a 1.7% TAE agarose gel at 100V for 65 min. The results were compared against a positive control provided by a 10 ng gDNA template and a negative control provided by water. Upon passing the PCR quality control, the extracted gDNA was subjected to amplicon library construction using a two-step PCR.

The first step of PCR was conducted using KOD-Multi & Epi-® (Toyobo) to amplify the targeted 16s rRNA genes at V3 and V4 regions. Forward and reverse primers (5′-CCTACGGGNGGCWGCAG and 3′-GACTACHVGGGTATCTAATCC) were applied respectively. The second step of PCR attached dual indices and Illumina sequencing adapters to the PCR products using Illumina Nextera XT Index Kit v2. PCR clean-up was carried out at the end of each PCR stage using AMPure XP beads and 80% ethanol. The libraries were then normalised and proceeded to sequence using Illumina MiSeq (USA) platform at 300 PE. Finally, the raw output data produced were processed using bioinformatic tools and analysed for operational taxonomic units (OTUs) clustering, taxonomic assignment, diversity assessment, and statistical analyses.

3. Results and discussion

3.1. Pharmaceutical compounds removals

The analysis of pharmaceutical compounds revealed that the three pharmaceutical compounds assessed in this study have different degrees of removal. Fig. 1 depicts the total removal as well as the degree of biotransformation and sorption of CAF, GCZ, and PRZ in Conditions I-IV.

In Condition I, GCZ was poorly removed, and only a total of 11 ± 6% removal was achieved. The removal efficiency of GCZ was comparatively higher at 78 ± 3% in Conditions II and III, while the GCZ initial concentration of 1000 μg L^{-1} in Condition IV resulted in 83 ± 4% removal. The higher removal efficiencies were relative to the initial GCZ

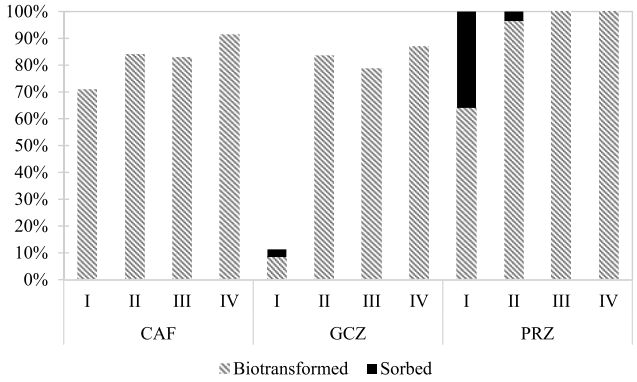

Fig. 1. Degree of biotransformed and sorbed CAF, GCZ, and PRZ in Conditions I-IV

concentration in respective experimental conditions. From the sludge analysis, it was discovered that GCZ was mildly sorbed to solids at 3% in Condition I, while no GCZ was detected in the solid samples in the other three conditions. It is also worth noting that the residual GCZ concentration in Conditions I-III was within a similar concentration range (88.37 ± 11.95 μg L^{-1}), while the residual GCZ in Condition IV was nearly two times the residual GCZ concentration in the earlier conditions. Petrie et al. [32] previously reported the persistency of GCZ in the application of horizontal sub-surface flow constructed wetlands. However, the removal of GCZ in this study was consistent with the outcome of a separate anaerobic incubation period study that recorded 83% of GCZ removal at the end of their experiment [26].

Compared to GCZ, the removal of CAF was much higher in Condition I, at 71 ± 6%. The removal of CAF was almost at the same level (84 ± 5%) for Conditions II-IV. No CAF was detected in the solid samples at all conditions. This outcome is comparable to the biotransformation recorded in previous batch experiments [26,33] and in anaerobic membrane reactor experiments at trace level concentration [34] and as sole substrate [35].

For PRZ, the concentration of the compound was below the LOQ in most effluent samples, except in the early stages of Condition IV (10.28 ± 0.12 μg L^{-1}). The analysis of PRZ in the sludge, however, revealed that 36% and 4% of PRZ concentration in Conditions I and II was sorbed to the solid phase. The concentration of PRZ in the sludge was below the LOQ in Conditions III and IV, respectively. The results from this study corresponded to the rapid removal of PRZ in the previous study by Azizan et al. [26].

Overall, the UASB bioreactor was capable of removing more than 70% of pharmaceutical compounds in all conditions except for GCZ in Condition I. The removal performance was not hindered and was able to remove a higher fraction of the compounds despite being present at higher concentrations in the latter experiments. This outcome may be contributed by the adaptation of the inoculum to the compounds. It was also inferred that a prolonged treatment period for each condition would not further improve the removal efficiencies as the threshold limit of the capability of the bioreactor to achieve lower residual concentrations could not be improved. Table 2 compares the removals recorded in this study to those of the previous studies.

3.2. Biotransformation and sorption of CAF, GCZ, and PRZ

Biotransformation accounted for most of the compound removals, while sorption was not a major removal pathway for all three compounds in this study, despite sorption occurrences for GCZ in Condition I and PRZ in Conditions I and II. Additionally, the increase in the initial concentration of the compounds in the wastewater promoted the treatment to favour biotransformation rather than sorption.

The biotransformation performance of the pharmaceutical compounds in this study is mainly driven by the chemical structures of the respective compounds. For instance, CAF has electron donating group amine and amide, and nitrogen atoms in its molecular structure [34], which makes it favourable to biotransform under anaerobic conditions. Meanwhile, the lower biotransformation of GCZ relative to CAF in this study may be influenced by the electron withdrawing group sulfonylurea in the chemical structure of GCZ. Sulfonylurea is commonly present in herbicides and is notable for its persistency when treated at neutral pH [37]. In the instance of PRZ, multiple electron donating groups within the piperazine structure of the compound resulted in consistently high biotransformation performance under methanogenic conditions, and at a greater magnitude compared to CAF and GCZ in this study.

Sorption was not a major removal route of the examined compounds in this study. While the hydrophilicity of CAF was consistent with the results from previous studies [34,38], this study has shown mild to no sorption for GCZ and PRZ in the UASB reactor. When sorption was relevant in Conditions I and II, PRZ displayed a

higher sorption degree compared to GCZ. The degree of sorption was correlated with the log D value of the respective compounds (log $D_{GCZ\ pH\ 7} = 0.79$; log $D_{PRZ\ pH\ 7} = 1.22$), whereby the larger coefficient represented the greater distribution of the compounds in the solid phase. The operational pH in the neutral range may have also contributed to this outcome, favouring PRZ ($pK_a = 7.24$) to have more neutral species and have a greater affinity towards the negatively-charged sludge surface [39]. Consistent with the results by Azizan et al. [26], the dynamic changes in sorption degree for GCZ and PRZ did not cause any accumulation in the sludge, and their concentration amount sorbed to sludge was reduced as the experiment continued. The probable reason for this occurrence may be due to sorption being an intermediary medium for the biotransformation of hydrophobic compounds [34]. However, as good biotransformation performance was achieved in most conditions, the sorption effect may not determine the biotransformation performance [40]. Instead, the biotransformation was attributed to the presence of electron donating groups within the chemical structure of the compounds [27].

3.3. Anaerobic process performance

The assessment of the UASB bioreactor performance verified that the process remained stable throughout its operation, and pharmaceutical concentrations from 100 to 1000 μg L^{-1} do not adversely impact the reactor stability. Fig. 2 provides the graphical representation of the bioreactor process performance. From the first day of introducing the pharmaceutical compounds in the wastewater flow until the end of the experiment, the COD removal efficiency was not significantly affected by the changing experimental conditions and was maintained at 93 ±

Table 2
Comparison of the removal efficiencies of CAF, GCZ, and PRZ achieved in this study and those in the literature.

Configuration	Wastewater type and pharmaceutical concentration	Operational condition	Removal efficiency (%)			Reference
			CAF	GCZ	PRZ	
Laboratory-scale UASB reactor	Synthetic wastewater, 0.1–1 mg L^{-1} of pharmaceutical mixture	Continuous operation with HRT 36–48 h	71–91	8–87	64 - > 99	This study
Anaerobic incubation	Synthetic wastewater, 1 mg L^{-1} of pharmaceutical mixture	90 days incubation under mesophilic condition	>99	83	>99	Azizan et al. [26]
Anaerobic incubation	Synthetic wastewater, 1 mg L^{-1} of pharmaceutical mixture	10 weeks incubation under mesophilic condition	>99	–	–	He et al. [33]
Laboratory-scale anaerobic membrane reactor	Coffee processing wastewater (caffeine as sole substrate), up to 2000 mg L^{-1} of pharmaceutical compound	Continuous operation with varying HRT 5–55 days under thermophilic (55 °C) condition	87.5 ± 5.3	–	–	Chen et al. [35]
Horizontal sub-surface flow constructed wetlands	Effluent from full-scale trickling filter, 83.8 ± 10.7 ng L^{-1} of pharmaceutical compound (actual occurring concentration)	Continuous operation with HRT of 14 ± 2 h for 12 months	–	13 ± 12 (result after 12 months)	–	Petrie et al. [32]
Laboratory-scale aerobic sequencing batch reactor	Raw municipal wastewater (105–215 mg COD L^{-1}), 1 mg L^{-1} of pharmaceutical mixture	30 days continuous operation with HRT 12 h,	–	39	41	Mat Zaini et al. [36]

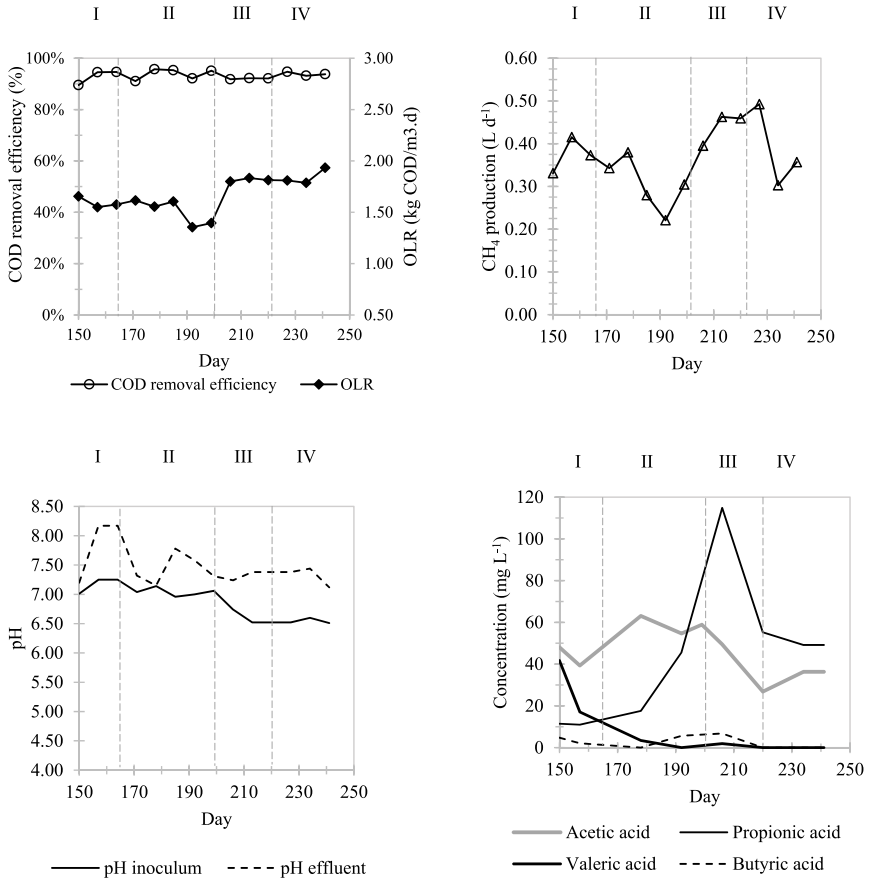

Fig. 2. UASB process performance for Conditions I-IV

2%. The inoculum pH was also within the neutral pH range (6.87 ± 0.29). Hydrogen gas was not detected in this study; thus, it is assumed that the whole hydrogen was present in a soluble form.

Although the differences are not too immense, it was rather evident that the biogas production fluctuated as the experimental conditions changed. In Condition I, methane production increased by 21% on average compared to the production prior to the introduction of the pharmaceutical compounds. However, the addition of 400 μg L^{-1} of the mixed pharmaceuticals returned the methane gas production level to the Baseline condition. The biogas production was recovered (0.184 ± 0.034 L CH_4 d^{-1}) due to the change of HRT from 48 h to 36 h in Condition III, but again it declined as 1000 μg L^{-1} of the mixed pharmaceuticals was introduced in Condition IV. It is also worth mentioning that the methanisation throughout the experiment did not significantly deviate from the Baseline values.

These results showed that the concentration of CAF, GCZ, and PRZ may be an influencing factor for biogas production. The pharmaceuticals displayed stimulatory behaviour towards methane production at lower concentrations (100 μg L^{-1}); however, it posed minor inhibition towards methane production at higher concentrations (400 μg L^{-1} and 1000 μg L^{-1}). In the study conducted by Fáberová et al. [22], CAF as an individual compound at concentrations of 10 and 500 μg L^{-1} mildly inhibited the methane generation. Since there is a lack of information about the stimulatory-inhibitory behaviour of GCZ and PRZ, these two compounds may possibly provide stimulation towards methane production to a certain extent and balance the total biogas production. The stimulatory behaviour was previously recorded for the psychostimulant carbamazepine and non-steroidal anti-inflammatory drugs (NSAIDs) diclofenac and ibuprofen, achieving up to an additional 60% of total methane production [22].

Further investigation on the biogas production resulted in no significant correlation between the utilisation of the three compounds and the methane yield throughout Conditions I-IV ($p > 0.05$) as shown in Fig. 3. Instead, the methane yield corresponded to the overall substrate utilisation in the UASB reactor ($p < 0.05$). This indicates that the methane yield was independent of the biotransformation of CAF, GCZ, and PRZ at concentrations below 1 mg L^{-1} and proved the theory of co-metabolic reactions between the biotransformation of these compounds and reduction of organic constituents present in the wastewater stream [40].

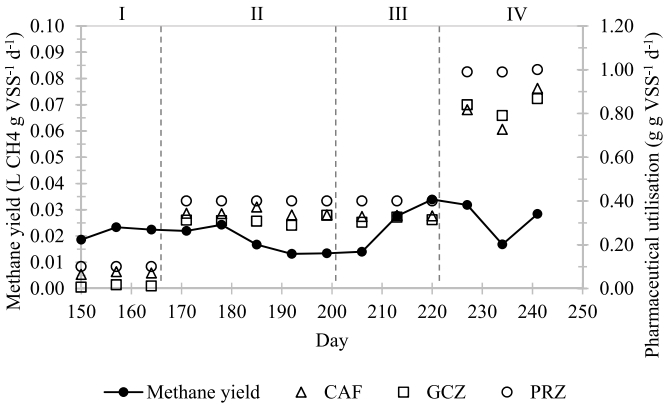

Fig. 3. Utilisation of pharmaceutical compounds and methane yield throughout Conditions I-IV

Based on the total VFA production, the concentration of VFA correlated with the biogas generation. VFAs were observed to be the most affected parameter in this study and provided an indication that the biotransformation of the pharmaceutical compounds influenced the acidogenesis process. The fluctuation in the acetic acid production was recorded between Conditions I and IV. While Condition II favoured the acetic acid production (58.89 ± 5.93 mg COD L^{-1}) more than Condition I (43.70 ± 6.17 mg COD L^{-1}), the production of acetic acid in Conditions III and IV dropped by 55% and 41% with respect to Condition II. Between the Baseline and Condition I, the propionic acid production remained below 12 mg COD L^{-1}. As Condition II experiment was ongoing, the propionic acid concentration increased gradually and reached its peak (115 mg COD L^{-1}) in the early stages of Condition III. The propionic acid production later declined and was maintained at approximately 50 mg L^{-1} until the end of Condition IV. In the case of valeric acid, its production was reduced to an insignificant level from the introduction of the mixed pharmaceuticals until the end of the experiment. There were also not many changes in butyric acid production except between the end of Condition II and the beginning of Condition III; however, its concentration remained below 10 mg COD L^{-1}.

In the study of antibiotics removal in anaerobic treatment, pharmaceutical compounds were found to promote the production of VFAs, notably acetic acid and propionic acid, as reported in this study. These VFAs increased significantly in the presence of sulfamethoxazole [17], tetracycline [17,41], and ciprofloxacin [16], as well as for the mixture of sulfamethoxazole, tetracycline, and erythromycin [18]. These results were attributed to the growing abundance of fermentative bacteria related to the production of these VFAs [16]. The authors in Ref. [16], however, examined the pharmaceutical compounds at concentrations of more than 1 mg L^{-1}, and the effect on the VFA production was considerably higher than the impacted VFAs in this study.

3.4. Shift in microbial diversity

3.4.1. Diversity assessment

The alpha- and beta-diversity of the samples were first calculated to assess the diversity. Alpha-diversity quantifies the richness or abundance of species within the same sample, whereas beta-diversity compares relative OTUs between multiple samples [42]. According to the result of alpha diversity, the presence of the pharmaceutical compounds effectively enriched and increased the abundance of OTUs. This was revealed through the calculation using the Chao1 and Shannon methods, which evaluate the species abundance and richness, respectively [42]. Additionally, the result of beta-diversity using the PCoA method showed that the diversities of OTUs in Conditions II and IV were similar to each other but deviated from the Baseline sample. The results of the diversity calculations are presented in Table 3.

3.4.2. Taxonomical unit information

The results of OTUs were further compared according to their compositions in the respective samples. As provided by the diversity calculations, both archaeal and bacterial composition were significantly shifted from Baseline to Conditions II and IV.

In the archaeal composition (Fig. 4), the presence of pharmaceutical compounds shifted the abundance from aceticlastic methanogens to hydrogenotrophic methanogens. Aceticlastic methanogens reduce acetate to produce methane and carbon dioxide, while hydrogenotrophic

Table 3
Alpha- and beta-diversity for Baseline, Conditions II and IV sludge.

Condition	Alpha-Diversity		Beta-Diversity
	Chao1	Shannon	PCoA
Baseline	690.27	3.85	0.459, 0.204
Condition II	817.53	4.48	−0.090, −0.269
Condition IV	877.40	4.21	−0.009, −0.269

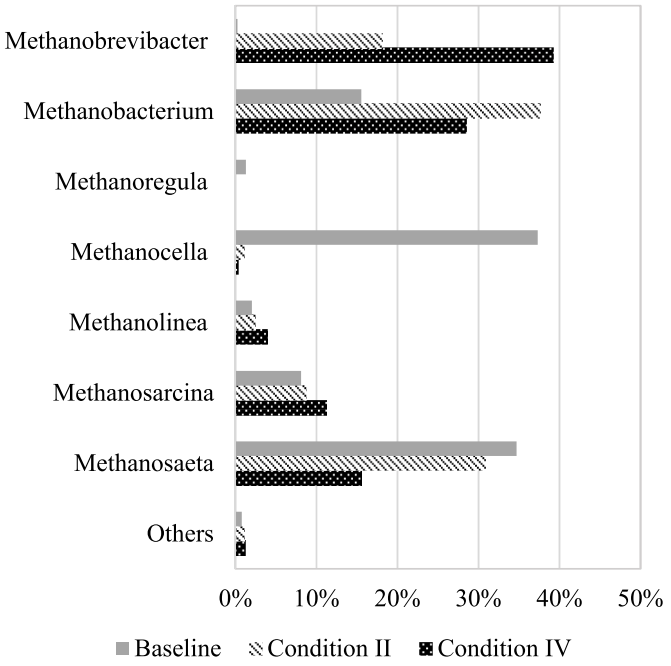

Fig. 4. Archaeal composition in Baseline, Condition II, and Condition IV samples.

methanogens grow on carbon dioxide and hydrogen to generate methane [43]. The abundance of *Methanosaeta* in the Baseline (35%) was decreased slightly in Condition II (31%), and further reduced in Condition IV (15%). *Methanosarcina,* which may act as an aceticlastic methanogen or hydrogenotrophic methanogen, fluctuated between 8 and 11% of the total archaeal genus composition. Considering that *Methanosaeta* grows strictly on acetate [8], its abundance is proportional to the production of acetic acid throughout the experiment. Comparatively, *Methanosarcina* was not as sensitive to the change in acetic acid as *Methanosaeta* probably due to the dominance of *Methanosaeta* as an aceticlastic methanogen in the bioreactor [44].

The pharmaceutical compounds were discovered to stimulate the growth of *Methanobrevibacter* and *Methanobacterium* from Baseline (0% and 16%) to Condition II (18% and 38%) and Condition IV (39% and 29%). In return, *Methanocella*, which was abundant in the Baseline (37%), became insignificant upon the introduction of the compounds. Based on the growth of these hydrogenotrophic methanogens, it is evident that the propionic acid and possibly a portion of acetic acid were oxidised to carbon dioxide and hydrogen as the substrates for these species, rather than directly or syntrophically converting to acetate for the aceticlastic pathway [43]. Previously, *Methanobrevibacter* species were discovered to grow in the presence of antibiotic oxytetracyline (20–80 mg L^{-1}) during the anaerobic treatment of cow manure [45], while *Methanobacterium* grew under the influence of antibiotic ciprofloxacin (0.5–50 mg L^{-1}) during the anaerobic incubation of sewage sludge [16].

Phyla Firmicutes was dominant among other bacteria in all conditions and became enriched as the experiment continued from Baseline (23%) to Condition II (34%) and Condition IV (37%). Spirochaetota was the second most abundant phyla in the Baseline sample (27%), but its abundance declined in Condition II (8%) and Condition IV (16%). The reduction in abundance was also recorded for Acidobacteriota from 13% in the Baseline sample to an insignificant amount in Conditions II and IV (1%). Instead, the proportion of Bacteroidota surpassed other phyla in Condition II (31%) and Condition IV (27%). Phyla Desulfobacterota, which consists of mostly the genus *Desulfovibrio,* also grew significantly from only 2% in Baseline sample to 8% in Condition II and 4% in Condition IV. Fig. 5 depicts the bacterial compositions in the samples.

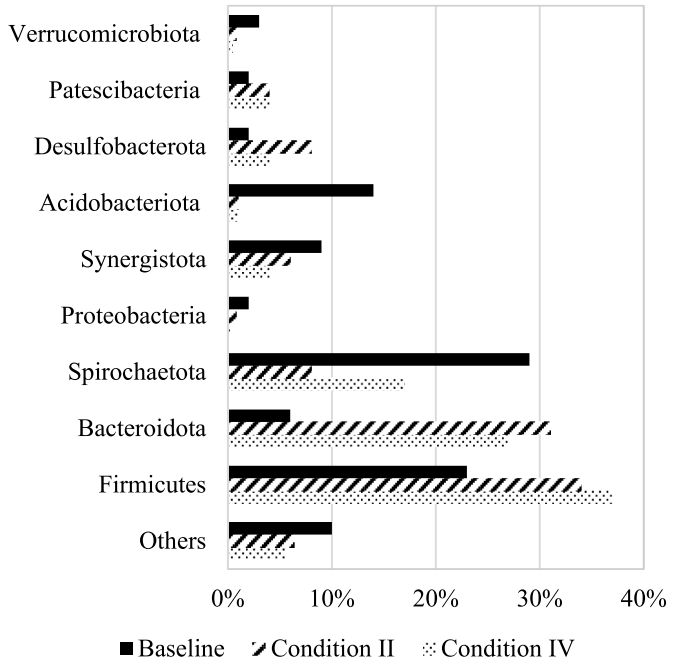

Fig. 5. Bacterial composition in Baseline, Condition II, and Condition IV samples.

Firmicutes and Bacteroidota have been linked to the degradation of a variety of organic matters for VFA production [46]. Among the enriched Firmicutes in Condition II of this study include genera *Clostridium sensu stricto* and *Oscillibacter* of class Clostridia, which may either be important acetogens [47] or syntrophic acetate-oxidising bacteria coupled with hydrogenotrophic methanogenesis [48]. In Condition IV, a significant growth was recorded for the genera *Megasphaera* and *Pectinatus* of the class Negativicutes, which are related to the production of various acids, including acetic and propionic acids [49]. Meanwhile, the enrichment of Bacteroidota in this study may be attributed to the pharmaceutical compounds as similar enrichment was recorded during the removal of ciprofloxacin [16], carbamazepine [50], and diclofenac [51]. Similarly, the growth of sulfate-reducing bacteria *Desulfovibrio* was previously connected to the biotransformation of various organic pollutants [52] and enriched in the removal of ciprofloxacin [16].

The results of the microbial assessment justified the variation level of VFAs production at each condition based on the growth or inhibition of fermentative bacteria. The change to the abundance of fermentative bacteria also provided an indication that these communities may be responsible for the biotransformation of CAF, GCZ, and PRZ in the UASB bioreactor, as claimed by He et al. [53] for the removal of amide pharmaceuticals in their study. Additionally, these bacteria affected the methanogenic pathway and seemed to divert some of the methanogenesis processes from aceticlastic to hydrogenotrophic pathway. As the growth of hydrogenotrophic methanogens thrived in the presence of the pharmaceutical compounds, it can be assumed that the methane gas generation by these methanogens made up for the loss of methane gas due to the reduction of aceticlastic methanogens.

4. Conclusions

The application of the UASB bioreactor to this study successfully removed up to 87–99% of CAF, GCZ, and PRZ, mainly through biotransformation. UASB operation remained stable with consistent methane yield throughout the experiment. However, under the influence of the three compounds, correlations were made between key anaerobic performance indicators and microbial diversity. It was revealed that the dominance of hydrogenotrophic methanogens over the aceticlastic compounds caused fluctuations in the methane gas production. Meanwhile, the favourable production of acetic acid and propionic acid corresponded with the shift of hydrolytic and fermentative bacteria compositions in the UASB reactor. These changes proved that key anaerobic performance indicators were susceptible to the presence of CAF, GCZ, and PRZ at the examined concentration level. This study also revealed that an actual continuous anaerobic process would continuously produce biogas for energy recovery despite the diversity shift of the methanogens in the process, in comparison to the inhibitory effect when examined in batch experiments. To the best of our knowledge, this is the first report of the PRZ removal in an anaerobic bioreactor.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biombioe.2022.106511.

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