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# Can anaerobic intermediate stages affect the biotransformation and sorption of pharmaceutical compounds?

Nur Alyaa Zahida Azizan[a], Ali Yuzir[b,*], Norhayati Abdullah[b], Fouad Fadhil Al-Qaim[c]

[a]*Malaysia-Japan International Institute of Technology (MJIIT), Universiti Teknologi Malaysia, Jalan Sultan Yahya Petra, 54100 Kuala Lumpur, Malaysia, Tel. +60193410143; email: alyaa1988@graduate.utm.my*
[b]*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, Tel. +60322031581; Fax: +60322031266; email: muhdaliyuzir@utm.my*
[c]*Department of Chemistry, Faculty of Sciences for Women, University of Babylon, Hilla, Iraq, Tel. +9647814042049; email: fouadalkaim@yahoo.com*

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**ABSTRACT**

Batch experiment was conducted to investigate if anaerobic intermediate stages can affect biotransformation and sorption performance of selected pharmaceutical compounds (stimulant caffeine (CAF), anti-diabetic drug gliclazide (GCZ) and anti-hypertensive drug prazosin (PRZ)). The outcome revealed that CAF was removed solely via biotransformation and followed the methanogenic pathway. Sorption was significant during the fermentation stage for hydrophobic GCZ and PRZ although both compounds continued to biotransform in the later stage of incubation. While more than 95% removal of all compounds was achieved after 30 d of incubation under anaerobic mesophilic condition, further incubation to Day 90 resulted in re-occurrences of GCZ and PRZ in the aqueous and solid phase. Biotransformation of hydrophilic and hydrophobic compounds could not be represented in the same kinetic model considering sorption and retransformation displayed significant effect to the model. This study also reported the first removal of GCZ and PRZ under anaerobic condition.

*Keywords:* Anaerobic digestion; Pharmaceutical compounds; Biotransformation; Sorption; Removal efficiency

## 1. Introduction

Occurrences of pharmaceutical compounds in water environment are currently a growing concern. Pharmaceutical compounds have been regarded as micropollutants and among the compounds of emerging concerns as they are often recalcitrant [1] and may potentially bioaccumulate in the ecosystem over a long period of continuous emission [2–4]. Previous occurrence studies in the Malaysian water environment revealed detection of pharmaceutical compounds at trace level concentration between ng/L and μg/L in treated effluents and river tributaries [5–7]. Among the detected compounds include stimulant caffeine (CAF), anti-diabetic drug gliclazide (GCZ) and anti-hypertensive drug prazosin (PRZ) [5–7].

CAF is a methylxanthine derivative, commonly consumed in Malaysia through beverages such as coffee and energy drinks [8,9]. GCZ is a sulphonylurea derivative prescribed to treat patients with type 2 diabetes mellitus [10]. PRZ is a piperazine derivative alpha-blocker which is consumed for the treatment of hypertension in Malaysia [11,12] as well as for the treatment of post-traumatic stress disorder [13] and erectile dysfunction [14]. Both GCZ and PRZ were recorded as the highest consumed drugs in their

* Corresponding author.

respective therapeutic groups and ranked within the top 50 most consumed drugs in Malaysia between years 2011 and 2016 [11,12].

CAF, GCZ and PRZ were present in treated effluent of conventional aerobic wastewater treatment processes that receive inflow of hospital wastewater and municipal wastewater [5–7]. Based on these reports, the treatment of these three compounds resulted from negative, medium to high removal efficiency within the same processes [5–7]. This provided evidence that the existing treatment processes were not designed to remove these compounds equally and posing even more concerns the negative removals. Consequently, the existing convention treatment processes became the pathway of the compounds to enter the water environment.

While conventional approaches pose disadvantages for the treatment of pharmaceutical compounds, biological treatment is still highly relevant as the main wastewater treatment technology. Anaerobic digestion is one of biological treatments that have been actively investigated in recent decade for its ability to remove pharmaceutical compounds from wastewater. Application of anaerobic process is favourable for reasons such as its ability to treat different types of wastewater at various organic loadings, operate with low dependency on energy and can potentially generate biogas for energy recovery [15,16]. The process is also feasible for the actual implementation as it is cost-efficient [17], independent of external resources, while simultaneously minimises the risk of by-products generation that may form through reaction with chemical additives [18].

It was previously reported that the degree of pharmaceutical compounds removal varied significantly ranging from excellent to poor removal, even when treated within the same anaerobic treatment study [19–22]. The assessment of individual compounds revealed that the removal performance was compound-specific based on their physico-chemical characteristics. This factor is critical in determining the behaviour of the compounds in anaerobic treatment such as their biodegradability, hydrophilicity and preferable removal pathway [23,24]. Under anaerobic condition, pharmaceutical compounds are commonly removed through biotransformation and/or sorption to anaerobic biomass. Unless the compounds are recalcitrant or highly hydrophobic, biotransformation is the most likely removal pathway in anaerobic treatment [25]. However, the variability of physico-chemical characteristics still affects the degree of biotransformation [26–29] and sorption to biomass [30–32].

In addition to physico-chemical characteristics, Alvarino et al. [33] addressed that sorption could also be one of the important factors influencing biotransformation. Previous studies recorded that sorption of pharmaceutical compounds to anaerobic biomass is directly related to hydrophobicity of respective compounds as well as the ambient pH of the treatment [20,34,35]. However, sorption is frequently evaluated upon completion of treatment rather than in the treatment intermediates and biotransformation can only be measured at the end of the experiment.

Taking into account the anaerobic process stages, the biological condition in the process will definitely change as the process develops from hydrolysis to fermentation and methanogenesis. Therefore, if biotransformation and sorption are assessed only at the end of the treatment, the results will not be able to provide an accurate picture of how the compounds behave in the treatment process throughout the changing anaerobic stages. It is important to assess both biotransformation and sorption of pharmaceutical compounds in the treatment intermediates to understand the behaviour of the compounds at each anaerobic stage and ascertain the key anaerobic parameters that can affect the removal pathways.

This study aims to investigate the critical factors determining biotransformation and/or sorption of pharmaceutical compounds including physico-chemical characteristics of a compound and key anaerobic process parameters. To address this relationship as compound-specific, selected compounds for this study were CAF, GCZ and PRZ. Batch experiment was conducted under anaerobic condition and assessments were made on the biotransformation and sorption degree of each studied compound. To the best of knowledge, this is the first report of GCZ and PRZ removal in anaerobic treatment.

## 2. Methodology

### 2.1. Chemicals and materials

The selection of pharmaceutical compounds for this study was based on their different physico-chemical properties (Table 1) and environmental occurrences in the Malaysian water environment [5–7]. Standards CAF, GCZ and PRZ hydrochloride purchased from Sigma Aldrich (USA) are of high purity (≥99%). High performance liquid chromatography grade water with 0.1% formic acid in water and acetonitrile were supplied by Merck (USA). Ultrapure water was obtained from Thermo Scientific Smart2Pure (Sweden).

Individual and mixed standard stock solutions of the three pharmaceutical compounds were prepared in methanol at 1,000 mg/L, and stored at −20°C to avoid or minimise the standards degradation. Further dilutions were made using ultrapure water to achieve desired concentrations.

Glucose-enriched synthetic wastewater was generated with glucose $C_6H_{12}O_6$ (2,720 mg/L), peptone (800 mg/L), yeast extract (560 mg/L) and ammonium chloride $NH_4Cl$ (320 mg/L). Trace elements added into the wastewater were calcium chloride $CaCl_2$ (40 mg/L), magnesium sulphate $MgSO_4$ (40 mg/L), iron(II) sulphate $FeSO_4$ (32 mg/L) and potassium dihydrogen phosphate $KH_2PO_4$ (60 mg/L). Sodium bicarbonate $NaHCO_3$ was introduced to maintain the reaction mixture at pH between 6.5 and 7.5 for optimum anaerobic reaction. The compositions of the wastewater were reagent grade supplied by Merck (USA) except for yeast extract (Difco, USA).

Table 1
Physico-chemical properties of selected pharmaceutical compounds [36]

| Compound | Molecular weight $M_w$ (g/mol) | p$K_a$ | log$K_{ow}$ | $s$ (mg/mL) |
|---|---|---|---|---|
| CAF | 194.191 | 10.4 | −0.07 | 11.0 |
| GCZ | 323.411 | 4.07 | 2.60 | 0.190 |
| PRZ | 383.401 | 7.24 | 1.30 | 0.693 |

Anaerobic digested biomass was sampled from a full-scale anaerobic mesophilic continuously stirred-tank reactor located in Kuala Lumpur. Upon sampling, the biomass was kept at 4°C before it was utilised as the inoculum for the batch experiments. The biomass was not acclimatised to the studied pharmaceutical compounds prior to the commencement of the experiment.

### 2.2. Experimental set-up

Batch experiments were conducted to examine the performance of pharmaceutical compounds removal under anaerobic mesophilic condition. Initially, the inoculum was incubated overnight at 37°C. Synthetic wastewater spiked with 1 mg/L of mixed standards solution was added to the inoculum at 50:50 (v/v) to give a total reaction volume of 120 mL in 250 mL air-tight glass bottles. Headspace was introduced to cater biogas production within the bottle. Each bottle was then purged with nitrogen gas for 5 min and sealed with butyl rubber stopper to remove the presence of oxygen and keep the reaction strictly anaerobic. Aluminium foil was used to wrap the bottles to eliminate possible photodegradation.

Experimental control was conducted to assess the abiotic effect. Mixed standard solution of concentration 1 mg/L was added to 120 mL of ultrapure water. All reaction bottles were incubated in water bath at 37°C for 90 d. The incubation period was determined to maximise the period of pharmaceutical compounds reaction with the non-acclimatised biomass. Fig. 1 depicts the set-up of the experiment.

### 2.3. Analytical method

#### 2.3.1. Anaerobic process performance

The measurement of pH, temperature, soluble chemical oxygen demand (COD) (sCOD), total suspended solids (TSS), volatile suspended solids (VSS) and volatile fatty acids (VFA) was conducted in accordance to the Standard Methods [37]. pH meter OHAUS Starter 3100 (USA) was utilised to monitor pH and temperature. COD analysis was assisted by Hach High Range Plus Reagent vials (USA) with Hach DRB200 reactor and DR6000 spectrophotometer.

Analysis of VFA was done using ion chromatography (ICS 5000+, Thermo Scientific, USA). Liquid samples were initially pre-treated by filtration using 0.45 μm nylon membrane filter (Thermo, USA) prior to analysis. Sample of 4.5 mL was injected through 4 × 250 mm analytical column (Dionex, IonPac™ ASII-HC) and detected using conductivity detector (Dionex P/N 60-062433). Elution was made using eluent Dionex EGC III KOH cartridge and ultrapure water (UPW) was used as the carrier solution.

Gas chromatography-thermal conductivity detector Clarus® 690 GC (Perkin Elmer, USA) instrument was utilised to analyse the biogas composition in all reaction bottles. Headspace gas of 5 mL was drawn from each bottle using an air-tight syringe and taken for loop injection. Nitrogen served as a carrier gas and its flow rate was fixed at 30 mL/min. The column and detector temperatures were set at 170°C and 200°C, respectively.

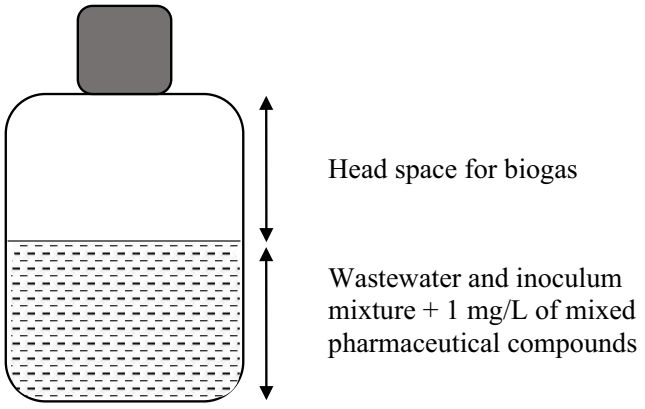


Fig. 1. Schematic diagram of the experimental set-up.

#### 2.3.2. Analysis of pharmaceutical compounds

Concentration of pharmaceutical compounds in aqueous and solid phase was determined by analysing each sample using ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) Waters ACQUITY UPLC-QDa instrumentation.

Initially, all samples were pre-filtered using 0.2 μm GH Polypro (GHP) syringe filter (Waters, USA) and transferred to borosilicate glass vials for analysis. Separation of the compounds (CAF, GCZ and PRZ) was conducted by directly injecting 30 μL of sample analyte through CORTECS® C18 column (2.7 μm 4.6 × 50 mm, Waters, Ireland). Mobile phases A and B for the elution were 0.1% formic acid in water and acetonitrile, respectively. The elution begins at 5% B and then increased linearly to 90% B for 4 min, then remained isocratic for 2 min. Next, the elution was returned to 5% B immediately and maintained at isocratic condition for another 2 min. All compounds were detected in positive ionisation mode using selective ion recording channels set at respective masses *m*/*z* of targeted compounds which are 195.09 *m*/*z* (CAF), 324.21 *m*/*z* (GCZ) and 384.25 *m*/*z* (PRZ). Results obtained from the analysis were analysed using Waters Empower 3 Software Build 3471.

Calibration curves for CAF, GCZ and PRZ were derived from standards injection at concentrations 10, 30, 50, 100, 300, 500 and 1,000 μg/L. The three compounds have calibration curves with the coefficient of determination $R^2 \geq 0.99$ (Relative standard deviation (RSD) ≤ 0.3%, $n = 6$). Recovery of the pharmaceutical compounds in wastewater was then evaluated based on the actual concentration quantified compared to the theoretical value of 1,000 μg/L of the mixed standards spiked into wastewater, directly injected through the liquid chromatography column. The recoveries of CAF, GCZ and PRZ were 70.1%, 66.2% and 68.1%, respectively.

Concentration of pharmaceutical compounds in biomass or solid phase was analysed using ultrasonic solvent extraction. Biomass from the reaction bottles were separated and 5 mL of the solids was drawn into a separate tube. A volume of 2.5 mL ultrapure water was added to the solids and centrifuged for 10 min at 3,700 rpm. The liquid phase was decanted and 5 mL of acetonitrile was added to the solid phase. The mixture was mixed with the assistance of the vortex mixture before it was sonicated for 20 min. Then, the mixture was centrifuged again for 10 min at 3,700 rpm.

The mixture was filtered through 0.2 μm GHP syringe filter and the filtrate was analysed using UPLC instrument following the procedure as previously stated.

### 2.4. Mathematical computations

Removal efficiency of the pharmaceutical compounds from the aqueous phase was calculated using Eq. (1). $C$ is the concentration of the compounds at each sampling period and $C_0$ is the initial concentration introduced in the reaction.

$$\text{Removal efficiency}\left(\%\right)=\left(1-\frac{C}{C_0}\right)\times 100 \tag{1}$$

The degree of sorption was also evaluated by comparing the initial concentration to the concentration of the compounds in solids, $C_s$ as per Eq. (2).

$$\text{Degree of sorption}\left(\%\right)=\frac{C_s}{C_0}\times 100 \tag{2}$$

Ultimately, the amount of compounds biotransformed $C_{\text{bio}}$ (μg/L) at each sampling period was calculated using Eq. (3), whereby $C_{\text{sorp}}$ is the concentration of pharmaceuticals sorbed in solids, $C_{\text{abio}}$ is the concentration of compounds removed through abiotic reaction and $C_{\text{res}}$ is the residuals concentration present in the aqueous phase.

$$C_0=C_{\text{bio}}+C_{\text{sorp}}+C_{\text{abio}}+C_{\text{res}} \tag{3}$$

Comparison of biotransformation model was made by fitting the results to the pseudo-first-order [Eq. (4)] and second-order kinetic models [Eq. (5)]. Here, biotransformation rate constant $k_{\text{bio\_1}}$ was estimated using this model as represented by Eq. (4), with $C_T$ as the total concentration of each compound in both aqueous and solid phase at time $t$ (d). Eq. (4) is the pseudo-first-order kinetic model and represented by unit 1/time of reaction.

$$\frac{d[C_t]}{d\left[C_0\right]}=-k_{\text{bio\_1}}\cdot t \tag{4}$$

$$\frac{d^2\left[C_t\right]}{d\left[C_0\right]^2}=-k_{\text{bio\_2}}\cdot t \tag{5}$$

To measure the maximum capacity of sorption of the pharmaceutical compounds in the solids, linear isotherm was adopted [Eq. (6)]. The linear isotherm is commonly applied to estimate sorption of compounds in biological processes when present at trace concentration level [38–41]. $C_A$ is the concentration of compounds in the aqueous phase corresponded to maximum $C_s$ and TSS is the total suspended solids recorded at the same sampling time.

$$\log K_d=\log\frac{C_s}{C_A\cdot \text{TSS}} \tag{6}$$

## 3. Results and discussion

### 3.1. Anaerobic process performance

Assessment of the batch experiment verified that anaerobic digestion was performing well in the entire incubation period. No inhibitory effect was observed throughout the treatment either from VFA or the pharmaceutical compounds.

From the analysis of VFA, it is evident that fermentation was most active within the first 30 d. VFA detected in this study mainly comprised of propionic acid, butyric acid and acetic acid. Fig. 2 depicts high VFA production rate up till Day 14. Concentration of propionic acid peaked on Day 7 and further increased to its maximum on Day 14 at 13.8 mg/L.VSS. Butyric acid was also present between Day 7 and Day 14 although at a much lower concentration. Acetic acid was produced later on Day14 at 9.16 mg/L.VSS. By Day 30, fermentation was completed in the absence of VFA. The production of VFA was also correlated with the production of biogas. More than 40% of methane was recorded by Day 7 and further increased to a maximum of 56% throughout the incubation period.

The presence of propionate and butyrate in the first 7 d of incubation indicated that the initial conversion of biogas was contributed by hydrogenotrophic methanogenesis [42,43]. Methane was subsequently produced through simultaneous hydrogenotrophic methanogenesis and aceticlastic methanogenesis from the conversion of all VFA [42,43]. The visibility of acetate on Day 14 with respect to the production of methane also indicated that aceticlastic methanogenesis is a slower process compared to hydrogenotrophic methanogenesis, agreeable to the findings by van Lier and Zeeman [44].

### 3.2. Pharmaceutical compounds removal

Analysis of the pharmaceutical compounds showed no significant photodegradation during the experiment. Thus, the abiotic effect was considered negligible in this study. Fig. 3 depicts the removal efficiencies of each compound under anaerobic digestion throughout 90 d of incubation.

Removal efficiencies of the three compounds in anaerobic digestion vary significantly throughout the incubation period. Almost half of the initial CAF concentration in

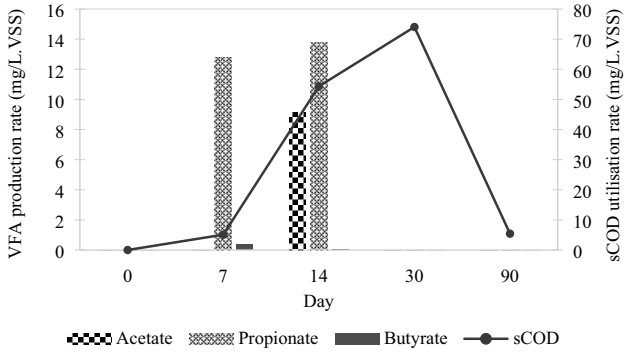


Fig. 2. VFA production rate and sCOD utilisation rate throughout 90 d of incubation.

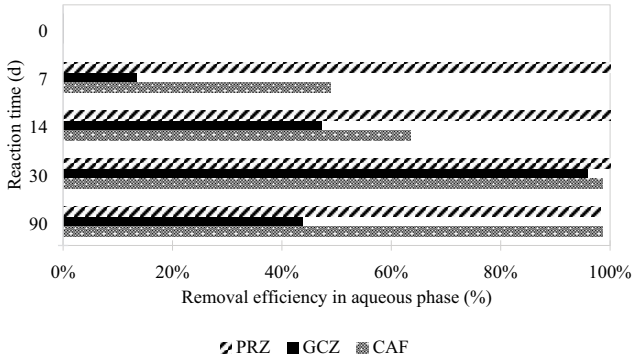


Fig. 3. Removal efficiencies of pharmaceutical compounds throughout incubation period under anaerobic condition.

wastewater was successfully removed by Day 7 and required at maximum 30 d for the concentration in the aqueous phase to reach below method detection limit (MDL).

The removal of GCZ has a similar trend to CAF removal although at a slower rate. Almost 50% of GCZ removal was achieved only upon reaching Day 14. The removal consequently improved up to 96% by Day 30. However, incubation after 30 d did not maintain the performance as GCZ removal surprisingly dropped to 56% on Day 90, with the sudden increment of GCZ concentration in the aqueous phase.

PRZ, in contrast, was removed rapidly within the first 7 d of incubation. Its concentration in the wastewater was consistently below MDL at each sampling period. Only on Day 90, about 1% of the initial PRZ concentration resurfaced in the aqueous phase.

The results of the removal performance provided information that incubation of up to 30 d under anaerobic condition utilising non-acclimatised anaerobic biomass was sufficient to achieve good removal efficiencies. Continuation of incubation to 90 d shown evidence of possible retransformation of GCZ and PRZ by-products to its parent compounds.

### *3.3. Degree of biotransformed and sorbed pharmaceutical compounds*

Pharmaceutical compounds removal was further assessed by evaluating the degree of sorption and biotransformation of respective compounds at each sampling period. Fig. 4 represents the degree of biotransformation and sorption of respective compounds throughout 90 d of incubation. Hydrophobicity of each compound was estimated by calculating the $\log K_d$ values.

No CAF was detected in the biomass throughout the treatment duration, and $\log K_d$ could not be estimated for this compound. Hydrophilicity of CAF was also reported by Wijekoon et al. [35] and Reyes-Contreras et al. [45]. It was clear that sorption to anaerobic biomass was not a selected pathway for CAF, and therefore, the removal of CAF in this experiment was solely through gradual biotransformation up to Day 90. This study supported the findings by He et al. [46] who recorded complete biodegradation of CAF under methanogenic condition in anaerobic batch experiments replicating constructed wetland conditions. Biotransformation of CAF may occur through co-metabolism when present at trace level concentration [46] or direct metabolism when CAF is present as a sole substrate at very high concentration [47].

Contrary to CAF, removal of GCZ and PRZ was through both biotransformation and sorption. Biotransformation of GCZ occurred from the initial stage of the incubation. By Day 14, the amount of sorbed GCZ significantly surged and equal to the amount of biotransformed GCZ. Sorption of GCZ was also most significant at this time although less than 3% of GCZ concentration was detected again after 90 d of incubation. GCZ was classified to be mildly hydrophobic with $\log K_d$ value of 1.18. GCZ is a sulphonylurea, a structure which is present in other medicines and herbicides. A study by Zheng et al. [48] on sulphonylurea herbicides revealed that transformation of sulphonylureas may be triggered by chemical hydrolysis and less favourable at neutral pH. Additionally, sulphonylurea bensulfuron methyl was recorded to be removed by biodegradation and sorption under methanogenic condition [49], as per the result of this study for GCZ.

PRZ was the most hydrophobic compound among the studied compounds, having the highest $\log K_d$ value at 4.19. Biotransformation of PRZ amounted to more than 70% as early as Day 7, while the remaining PRZ was sorbed to the anaerobic biomass. Surprisingly, total biotransformed PRZ dropped with the increase of the sorption degree at Day 14. These results indicated that there were possibilities of PRZ metabolites retransformed to its parent compound and consequently sorbed to the biomass. Eventually, the sorbed PRZ biotransformed again in the later stage of incubation, leaving less than 3% of PRZ initial concentration remaining in the biomass. Despite the occurrences of compounds containing piperazine structure in the environment [50–52], there are currently limited references to anaerobic removal of these compounds. While de Graaff et al. [53] recorded that cetirizine, a piperazine compound had negative removal when treated in up-flow anaerobic sludge blanket reactor, and no significant correlation can be made with the outcome of PRZ removal in this study.

Hydrophobic compounds in this study, i.e., GCZ and PRZ, occasionally showed sorption/desorption and biotransformation/retransformation activities during the anaerobic experiment. These activities are the common contributors which resulted in negative removal of pharmaceutical compounds in wastewater treatment processes [54–56].

There were also arguments if sorption actually limits biotransformation potential of pharmaceutical compounds [57,58]. In this study, the biomass did not retain high concentration of GCZ and PRZ until the end of the experiment, with respect to the maximum occurrence of GCZ and PRZ in the biomass in the first 14 d. The sorption of these compounds to the biomass was not static with longer incubation period and the reduction of the sorbed compounds supported the assumption that sorption promotes the contact between the compounds and microorganisms for biotransformation activities [24,59,60], by being the intermediate pathway to biotransform the compounds. Longer incubation time as experimented in this study consequently biotransformed the sorbed GCZ and PRZ upon reaching 30 d of incubation.

In total, CAF, GCZ and PRZ were successfully removed by more than 95% through biotransformation compared

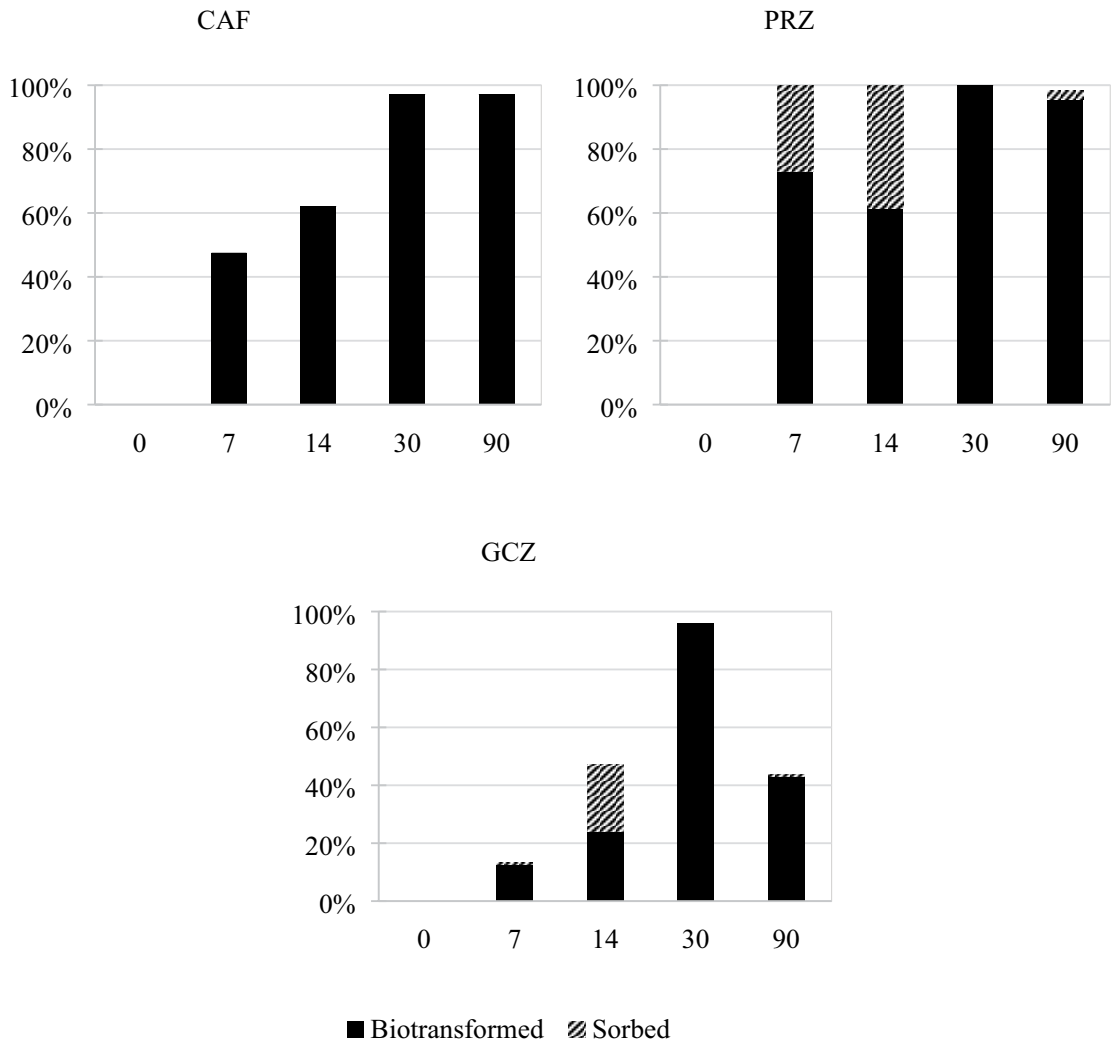


Fig. 4. Degree of biotransformation and sorption of respective compounds throughout incubation period under anaerobic condition.

to the initial spiked concentration by Day 30. Conversely, sorption/desorption and retransformation of GCZ and PRZ increased the occurrences of the compounds in both aqueous and solid phase at the end of the experiment. Final concentration of the compounds in wastewater effluent and anaerobic biomass at the end of the experiment is specified in Table 2.

### *3.4. Relationship of anaerobic intermediate stages and sorption of pharmaceutical compounds*

Removal performance of the compounds by sorption was compared with the stages of anaerobic digestion. It is worth noting that the pH maintained within the neutral range (pH = 7.03 ± 0.29) as regulated with the addition of $NaHCO_3$ throughout the incubation period as stated in Section 2.1.

Results revealed sorption was associated with fermentation activity during the incubation. This is because the sorption of GCZ and PRZ occurred only during the presence of VFA. Compounds with higher hydrophobicity may be more sensitive to the changing environment [34], hereby, the acidic environment provided through VFA production. Polarisation of the compounds induced by the $H^+$ ions may increase the compounds' attraction to the negatively charged biomass [61]. Thus, higher concentration of $H^+$ ions represented by the VFA concentration may intensify the polarisation and consequently achieving a higher degree of sorption.

Table 2
Concentration of pharmaceutical compounds in effluent and biomass after 90 d

| Compound | Concentration in effluent (μg/L) | Concentration in anaerobic biomass (μg/g) |
|---|---|---|
| CAF | <MDL | N.D. |
| GCZ | 372.4 | 3.922 |
| PRZ | 11.67 | 18.86 |

MDL, method detection limit; N.D., not detected.

Moreover, the pH control at neutral made sorption even more favourable to the neutral compound PRZ ($pK_a$ = 7.24) rather than the acidic compound GCZ ($pK_a$ = 4.07), as hydrophobicity increases when the ambient pH is nearest to its $pK_a$ value [34,35]. Total VFA production at 13.15 mg/L. VSS was sufficient to induce high PRZ sorption degree on Day 7. Polarisation of acidic compounds instead is more favourable in the acidic environment. This explained the need of an additional 40% of VFA concentration to achieve similar degree of GCZ sorption on Day 14, as to what was achieved by PRZ on Day 7 at neutral pH. GCZ and PRZ continued to be biotransformed rather than maintained in the biomass after fermentation was completed.

This study has shown that concentration of VFA during fermentation is certainly a major factor influencing the sorption of pharmaceutical compounds in anaerobic digestion, in addition to the physico-chemical characteristics of a compound at neutral pH. It is also a critical finding as it explained the reasoning behind the variation of sorption degree in anaerobic process. There is ambiguity, however, if high VFA concentration can trigger the retransformation of PRZ and increase the availability of the parent compound for sorption on Day 14. Should this be the case, VFA may be an important parameter in determining the reversibility of biotransformation activities in anaerobic treatment. This assumption needs clarification by carrying out further assessment on this subject.

### *3.5. Relationship of anaerobic intermediate stages and biotransformation of pharmaceutical compounds*

Biotransformation of pharmaceutical compounds is often associated with co-metabolism with methanogenic activity as these compounds often present in wastewater at trace concentration level [24,33]. To ascertain this relationship, the biotransformation results were fitted to the pseudo-first-order and second-order kinetic models. The results indicated that the rate of biotransformation of CAF was the highest in this study in both models, followed by GCZ and PRZ. Biotransformation rate constants for each compound can be referred to in Table 3.

Pomiès et al. [39] suggested that pseudo-first-order kinetic model can be generally adopted to visualise the biotransformation rate. In this study, only biotransformation of CAF fitted the pseudo-first-order model within the acceptable confidence level (α = 0.05), with biotransformation rate constant $k_{bio\_1}$ of 0.118 1/d. This result provided an indication that biotransformation of CAF is directly related to the methanogenic activity throughout the incubation period. The results for CAF also supported the evidence that the first-order model is suitable to represent biotransformation of hydrophilic compounds [24,39].

Gonzalez-Gil et al. [24] also reported that pseudo-first-order model may produce inaccurate representation, especially for hydrophobic compounds, given the fact that it resulted in large confidence interval values [24,60]. This study agreed with the statement, considering the poor fit of both hydrophobic GCZ and PRZ in the pseudo-first-order model. Instead, biotransformation of GCZ and PRZ was best fitted in the second-order model with $k_{bio\_2}$ of 0.00001 and 0.00035 L/μg d. While co-metabolism activity may be active for biotransformation of GCZ and PRZ, it is evident that the biotransformation of these compounds was significantly affected by sorption/desorption and retransformation activities.

Observations on behaviour of PRZ in anaerobic treatment in this study led to plausible explanations to this issue include (i) rapid PRZ biotransformation in the initial stage of incubation, (ii) possible retransformation of PRZ to its parent compound between Day 7 and Day 14, and (iii) high tendency of sorption within the first 14 d of incubation. The bi-phase biotransformation was also discovered by Zhang et al. [62] whereby estrogens were biotransformed quickly in the earlier stage and slower in the later stage of the experiment. Higher initial concentration of PRZ applied in this study may also prompt a different metabolic pathway, as to what was observed by Jewell et al. [63] in their study of trimethoprim biotransformation in activated sludge.

While the sorption of GCZ was not as profound as compared to PRZ, retransformation of GCZ was extremely high by the end of the incubation period. This occurrence was contradicted to co-metabolic pathway [64] and difficult to explain as there are limited findings that reported on metabolism involving retransformation of pharmaceutical compounds. Some authors have addressed the issue of complexity in establishing a model to predict the biotransformation of pharmaceutical compounds in anaerobic condition as it is evident that the model could not be represented [65,66].

Gonzalez-Gil et al. [24] also deliberated on the inclusion of reversible biotransformation factor in the model. Nevertheless, to simplify the model, comparison of pseudo-first-order and second-order kinetic models is sufficient to visualise that the pseudo-first-order model is best applied only when biotransformation is the sole pathway of the compound removal. Whereas, the second-order model may be considered to represent compounds which are influenced by sorption and possible retransformation activities.

Table 3
Statistical analysis of each pharmaceutical compound fitted to pseudo-first-order and second-order kinetic models (α = 0.05)

| Compound | Pseudo-first-order kinetic model | | Pseudo-second-order kinetic model | |
|---|---|---|---|---|
| | $k_{bio\_1}$ (1/d) | *P* value | $k_{bio\_2}$ (L/μg d) | *P* value |
| CAF | 0.118 | 0.02 | 0.00168 | 0.09 |
| GCZ | 0.00542 | 0.07 | 0.00001 | 0.04 |
| PRZ | 0.0291 | 0.06 | 0.00035 | 0.01 |

## 4. Conclusion

This study has successfully compared anaerobic treatment performance of three different pharmaceutical compounds with different physico-chemical characteristics. Hydrophilic compound CAF was solely removed through biotransformation and directly related to the methanogenic activity. Whereas biotransformation of GCZ and PRZ was affected by sorption and retransformation activities. Throughout the anaerobic incubation, the fermentation stage directly influenced the sorption degree of GCZ and PRZ, and consequently indirectly affecting the biotransformation performance of these compounds. These findings provided an understanding of the relationship between these compounds and critical anaerobic parameters which will be an important reference for future study in examining the continuous removal of CAF, GCZ and PRZ in anaerobic bioreactor.

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