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A pilot-scale anaerobic moving-bed biofilm reactor with PVA gel beads as media for the treatment of fish canning industry wastewater

Akarawin Jirasaranporn[a], Krittayapong Jantharadej[a], Kritchart Wongwailikhit[b], Chamorn Chawengkijwanich[c,e], Jenyuk Lohwatcharin[a,e,f] and Benjaporn Boonchayaanant Suwannasilp[a,d,e,f,*]

[a] Department of Environmental Engineering, Faculty of Engineering, Chulalongkorn University, Bangkok 10330, Thailand
[b] Department of Chemical Engineering, Faculty of Engineering, Chulalongkorn University, Bangkok 10330, Thailand
[c] National Nanotechnology Center (NANOTEC), National Science and Technology Development Agency (NSTDA), Pathum Thani 12120, Thailand
[d] Center of Excellence on Hazardous Substance Management, Chulalongkorn University, Bangkok 10330, Thailand
[e] Research Network of NANOTEC-CU (RNN), Chulalongkorn University, Bangkok 10330, Thailand
[f] Professor Aroon Sorathesn Center of Excellence in Environmental Engineering, Chulalongkorn University, Bangkok 10330, Thailand
*Corresponding author. E-mail: benjaporn.bo@chula.ac.th

KJ, 0000-0003-4579-7589; KW, 0000-0001-5493-259X; JL, 0000-0002-0286-2670; BBS, 0000-0003-4321-5370

ABSTRACT

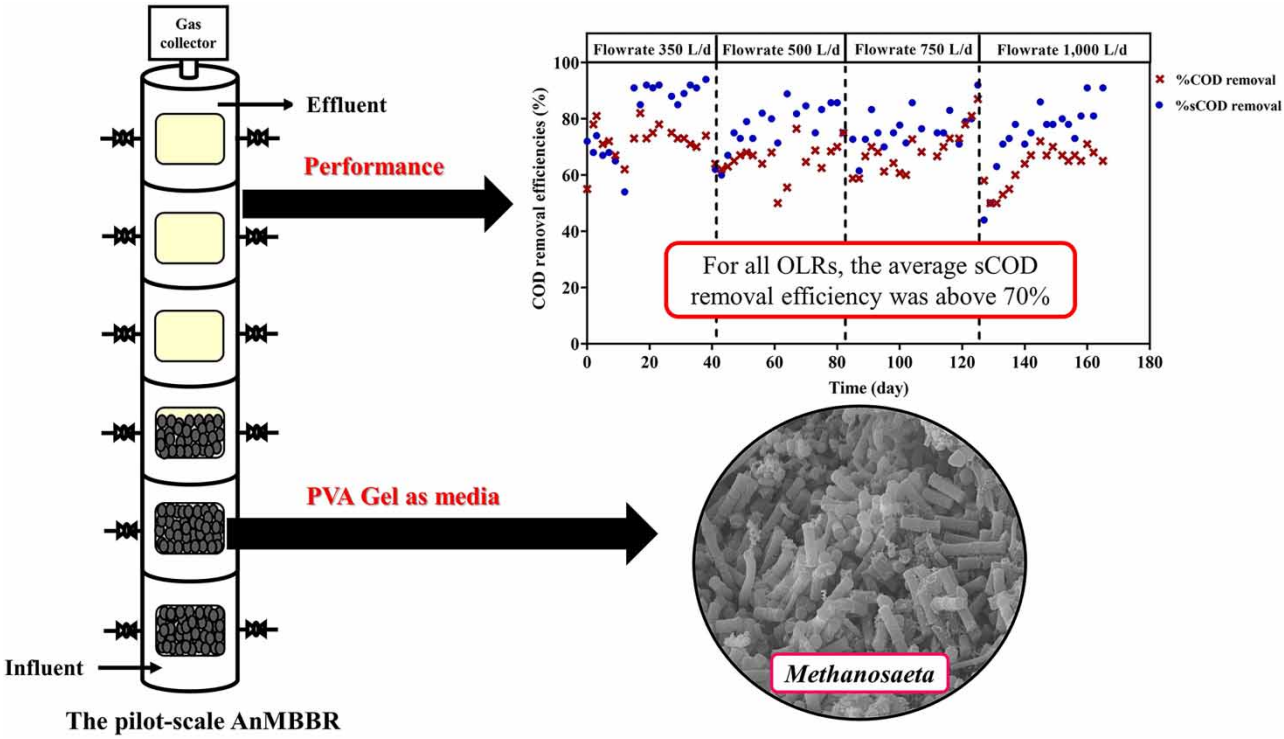
This research aims to investigate the performance of a pilot-scale anaerobic moving-bed biofilm reactor (AnMBBR) using PVA gels as media for the treatment of real wastewater from a fish canning factory. The chemical oxygen demand (COD) removal efficiencies at different organic loading rates (OLRs) were investigated at 3.0, 4.3, 6.5, and 8.7 kg COD/m^3 day by adjusting the flow rates to 350, 500, 750 and 1,000 L/d, respectively. The soluble COD removal efficiencies of the system at flow rates of 350, 500, 750, and 1,000 L/d were 81.0 $\pm$ 12.4%, 76.8 $\pm$ 8.2%, 74.7 $\pm$ 6.2%, and 70.6 $\pm$ 12.4%, respectively. According to the residence time distribution (RTD) tests at the highest and lowest flow rates, the mean residence times of both flow rates were significantly higher than the theoretical residence time, indicating very strong external recirculation inside the AnMBBR. The results suggest a 3-pass flow pattern through the AnMBBR. From 16S rRNA gene amplicon sequencing (MiSeq, Illumina) and scanning electron microscopy (SEM) analysis, *Methanosaeta*, acetoclastic methanogens, were the predominant microorganisms in the system. Most of the microorganisms were located within a 1.994 $\pm$ 0.266 mm depth from the PVA gel surface, with two distinct layers.

Key words: AnMBBR, food wastewater, pilot-scale, polyvinyl alcohol gel

HIGHLIGHTS

- The average sCOD removal efficiencies were above 70% for all OLRs (3.0–8.7 kg COD/m^3 day).
- The AnMBBR has a 3-pass flow pattern due to strong external recirculation.
- *Methanosaeta,* acetoclastic methanogens, were predominant in the system.

GRAPHICAL ABSTRACT



1. INTRODUCTION

Wastewater from the food industry generally contains high concentrations of organic compounds, which can lead to several environmental concerns if not treated properly. Anaerobic wastewater treatment is considered to be one of the most suitable approaches for the treatment of high-strength organic wastewater due to its cost effectiveness and the production of renewable energy in the form of biogas.

An anaerobic moving-bed biofilm reactor (AnMBBR) is a promising anaerobic wastewater treatment technology that can provide high sludge retention, which is an important factor contributing to high organic loading rates (OLRs). AnMBBRs use an attached growth process designed to cultivate microbes on the media to prevent biomass loss. There are several types of media commonly used in AnMBBRs with various sizes and shapes, which are mostly made of polyethylene (PE), high-density polyethylene (HDPE), or polypropylene (PP) (Mao *et al.* 2017).

Polyvinyl alcohol (PVA) gel is another material that has been used recently as media in AnMBBRs. PVA gel beads are synthetic media with a high specific surface area, which enables the cultivation of a large number of microorganisms, making them an interesting option for use in AnMBBRs (Rajpal *et al.* 2021). Due to their porous structures and large internal surface area, PVA gel beads were estimated to have an effective specific surface area of 2,500 m^2/m^3, which is much larger than their external specific surface area of 1,000 m^2/m^3 (Levstek *et al.* 2010). The effective specific areas of PVA gel beads were larger than those of other types of media (PE, HDPE, and PP), which have specific areas in the range of 200–1,200 m^2/m^3 (Barwal & Chaudhary 2014). The minute pores (10–20 μm) in the PVA gel beads could also serve as a protective environment for the microorganisms, thereby reducing the biomass sloughing from the media (Wang *et al.* 2018b). Moreover, due to a specific gravity close to that of water, PVA gel beads can be easily suspended in water (Hoa *et al.* 2006). In addition, since PVA gel is not biodegradable and is insoluble in water, it is durable for use in biological treatment systems. These advantages of PVA gel beads make them a promising media for use in AnMBBRs.

Many previous studies used PVA gel beads as media in various types of anaerobic bioreactors for the treatment of organic wastewater (Zhang *et al.* 2007, 2009; Wenjie *et al.* 2008; Khanh *et al.* 2011; Chaikasem *et al.* 2014, 2015; Jin *et al.* 2016; Yue *et al.* 2016; Jeong *et al.* 2017; Wang *et al.* 2018b). Synthetic organic wastewater (e.g., corn steep liquor, peptone–bonito

extract, ethylene glycol, molasses, synthetic high-strength particulate wastewater, and tapioca starch-based synthetic wastewater) was mostly used in the research. Only two previous studies investigated anaerobic treatment processes with PVA gel beads using real wastewater, i.e., domestic wastewater supplemented with food waste recycling wastewater (Jeong *et al*. 2017) and pharmaceutical wastewater mixed with simulated wastewater containing glucose (Wang *et al*. 2018b).

Furthermore, all previous studies on anaerobic bioreactors with PVA gel beads were performed at the laboratory scale. A pilot-scale test on this new technology is still lacking. The most comparable study to date is a pilot test using an upflow anaerobic sludge blanket (UASB) reactor with cylindrical anaerobic microorganism carriers (AMCs) as media, which share some common characteristics with PVA gel beads, for the treatment of textile wastewater with the main function of decolorization (COD removal efficiency of 37.0%; color removal efficiency of 50.3%) (Liu *et al.* 2020). Pilot-scale tests of AnMBBRs with PVA gel beads as media are still required before they can be applied to full-scale wastewater treatment plants, as they provide opportunities to monitor process stability in the presence of natural fluctuations in raw wastewater composition (Malmqvist *et al.* 1998) and help us anticipate potential problems that may arise in full-scale systems.

In addition, the microbial community is one of the most important components of anaerobic wastewater treatment. Complex microbial communities with synergy between different groups of microorganisms (e.g., acidogens, acetogens, and methanogens) are required for the successful operation of anaerobic bioreactors. Due to the distinct characteristics of PVA gels, e.g., internal minute pores, microbial communities on the PVA gel beads could be different from those on other types of media. Despite its importance, little information is currently available on microbial communities in anaerobic bioreactors with PVA gel beads (Khanh *et al.* 2011; Wang *et al.* 2018b; Pandey & Sarkar 2019). Microbial communities in PVA gel beads in two-stage anaerobic packed bed bioreactor systems were investigated using a culture-dependent technique (Pandey & Sarkar 2019). In UASB reactors with PVA gel beads as media, a bacterial community was examined using 16S rRNA gene amplicon sequencing (Wang *et al.* 2018b), whereas an archaeal community was investigated using a 16S rRNA gene clonal library (Khanh *et al.* 2011). However, complete information on microbial communities, including both bacteria and archaea, is still needed because their syntrophic interactions are critical in the anaerobic biodegradation of organic compounds. With advanced molecular techniques such as next-generation sequencing, detailed information on microbial communities can be obtained, which will improve our understanding of the microbial processes in AnMBBRs with PVA gel beads.

This study, therefore, aims to investigate the performance of a pilot-scale AnMBBR with PVA gel beads in the treatment of real wastewater from the food industry, specifically the fish canning industry, at various OLRs. Furthermore, the microbial communities in the PVA gel beads were identified using 16S rRNA gene amplicon sequencing (MiSeq). The localization, biofilm structure, and morphology of the microorganisms on the PVA gel beads were investigated using scanning electron microscopy (SEM). The residence time distribution (RTD) in the pilot-scale AnMBBR in the presence of PVA gel beads was also studied. To the best of the authors' knowledge, this is the first pilot-scale study of AnMBBR using PVA gel beads as media for the treatment of organic wastewater with comprehensive information on the microbial community and RTD. The results obtained in this study are greatly useful for the design and operation of full-scale AnMBBRs with PVA gel beads as media in the future.

2. MATERIALS AND METHODS

2.1. Pilot-scale AnMBBR system assembly

The pilot-scale AnMBBR in this study had a total volume of 288 L with a diameter of 0.35 m and a height of 3 m. It was installed at the wastewater treatment plant of a fish canning factory (Royal Foods Co., Ltd, Thailand). The reactor was separated into six zones, 0.5 m for each. In each zone, there were two ports on the opposite sides, with and without a screen, for sampling the PVA gel beads and water, respectively. The pilot-scale AnMBBR was connected to an inlet tank, an outlet tank, pumps, and pipes, as shown in Figure 1. A 1 m^3 inlet tank was used to prepare the influent by receiving raw wastewater from the fish canning factory after primary treatment, including screening, grease traps, and an equalization tank, which had a COD of 3,775 $\pm$ 844 mg/L. Supplementary Table S1 shows the composition of wastewater from the fish canning factory. The wastewater was supplemented with nutrients and sodium bicarbonate as a source of alkalinity according to Supplementary Table S2. A centrifugal pump (A) was used at a flow rate of 240 L/min to continuously mix the inlet tank. A metering pump (B) with a flow rate of 1,000 L/d and 5 m head was used to feed influent from the inlet tank to the reactor. Another centrifugal pump (flow rate of 9 m^3/min with 5 m head, C) was used for effluent recirculation.

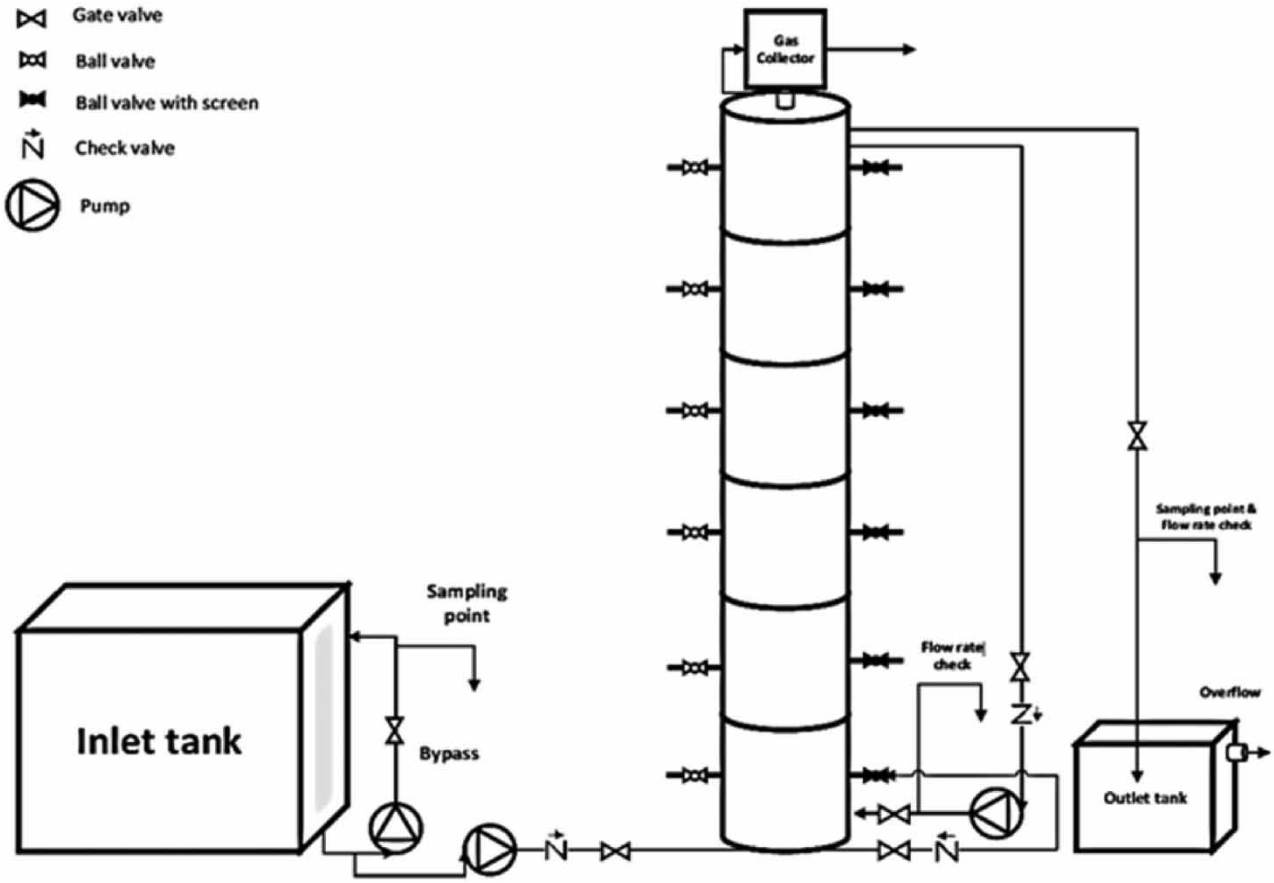


Figure 1 | Configuration of the pilot-scale AnMBBR system used in this study.

2.2. Incubation of PVA gel beads

PVA gel beads with a diameter of 4 mm (Kuraray Aqua Co., Ltd, Japan) were used as media in the pilot-scale AnMBBR. The specific density of PVA gel was 1.025 (Kuraray Aqua Co., Ltd, Japan). The PVA gel beads were incubated with UASB seed granules from the fish canning factory (Royal Foods Co., Ltd, Thailand). The UASB granules were broken into slurry before use as inoculum. Three 70-L batch reactors were used for PVA gel incubation with 35 L of PVA gel beads, 15 L of inoculum, and 15 L of wastewater in each tank. Nutrients and sodium bicarbonate (Supplementary Table S2) were added to the tanks and mixed thoroughly. Every 2 days during incubation, the supernatant was removed and replaced with an equal volume of wastewater containing nutrients and sodium bicarbonate. Incubation was continued for 1 month to promote the growth of microorganisms, which could be observed from a change in PVA gel color from light to dark (Wang *et al*. 2019).

2.3. Start-up of the pilot-scale AnMBBR with PVA gel beads

Incubated PVA gel beads were transferred into the pilot-scale AnMBBR at 30% of the reactor volume. Raw wastewater from the fish canning factory after primary treatment was continuously fed into the reactor at a flow rate of 350 L/d, which corresponded to an OLR of 3.0 kg COD/m^3 day. The initial upflow velocity was 0.5 m/h, and it was gradually increased to 1.5 m/h by adjusting the recirculation flow rate. All operating problems related to the reactor, pumps, pipes, and valves were solved during start-up.

2.4. Effect of the OLR on the performance of the pilot-scale AnMBBR

The performance of the pilot-scale AnMBBR with PVA gel as media was investigated at different OLRs, including 3.0, 4.3, 6.5, and 8.7 kg COD/m^3 day, by varying the influent flow rate at 350, 500, 750, and 1,000 L/d, respectively. OLRs were calculated from $OLR = Q \times COD/V$ using the actual COD concentrations in the real fish canning wastewater. The flow rate was increased to 1,000 L/d, corresponding to a hydraulic retention time (HRT) of 6.9 h, which is close to the HRT lower limit

in anaerobic bioreactors (6 h). For each flow rate, the pilot-scale AnMBBR was operated until reaching steady state, which took approximately 1.5 months. The upflow velocity was set at 1.5 m/h throughout the operation. COD, soluble COD (sCOD), total suspended solids (TSS), alkalinity, volatile fatty acids (VFAs), pH, and oxidation–reduction potential (ORP) in the AnMBBR were monitored regularly. The pH and ORP were measured using a pH meter (Mettler Toledo FiveEasy, Canada) and an ORP meter (Thermo Scientific ORLON4 STAR, Singapore), respectively. The concentrations of COD and sCOD were measured using the closed reflux method (APHA *et al.* 2005). The TSS was measured using the gravimetric method. Alkalinity and VFAs were monitored in the active zone or the PVA gel expansion zone, which was located between the second and third ports from the bottom of the reactor, using the titration method (APHA *et al.* 2005). Biogas production was measured by a gas counter.

2.5. RTD in the pilot-scale AnMBBR

The RTD in the AnMBBR reactor was investigated in the presence of PVA gel beads, in which microorganisms were abundantly grown on the PVA gel. Flow rates of 350 and 1,000 L/d were used to represent the minimum and maximum possible flow rates inside the reactor. The experimental setup was similar to that previously described. Influent was continuously fed into the reactor until a steady state was achieved. Afterward, a tracer (150 g/L NaCl) was injected into the reactor. The conductivity electrode was connected to a conductivity transmitter to monitor the tracer in the effluent until stable values were achieved. The mean residence time (t_m) of liquid was calculated based on the concentration of tracer as shown in Equation (1), where C_i is the tracer concentration at time i. The mean residence time was compared with the theoretical residence time, t_{theo}, calculated using Equation (2) to analyze the dead liquid portion inside each column, where Q_L is the liquid flow rate (Levenspiel 1999). The variance of t_m was computed using Equation (3) for further analysis.

$$t_m = \frac{\sum t_i C_i \Delta t_i}{\sum C_i \Delta t_i} \tag{1}$$

$$t_{theo} = \frac{V_L}{Q_L} \tag{2}$$

$$\sigma^2 \cong \frac{\sum (t_i - t_{mean})^2 \, C_i \Delta t_i}{\sum C_i \Delta t_i} = \frac{\sum t_i^2 C_i \Delta t_i}{\sum C_i \Delta t_i} - t_{mean}^2 \tag{3}$$

In addition, the exit age distribution ($E(t)$) was calculated from the tracer concentration profile, as shown in Equation (4). $E(t)$ was plotted as a function of time to analyze the liquid flow pattern using the compartment model as well as the tank-in-series model (Levenspiel 1999).

$$E(t) = \frac{C(t)}{\int_0^\infty C(t)\, dt}, \tag{4}$$

$E(t)$ as a function of time for the tank-in-series model, as expressed in Equation (5), was used to model the flow in the AnMBBR system, where N_{tank} is the number of tanks-in-series. Nonlinear regression was performed by comparing the dimensionless E-curve from the experiment with the tank-in-series model to obtain the optimum number of tanks-in-series and the residence time of each tank.

$$t_m \cdot E = \left(\frac{t}{t_m}\right)^{N_{tank}-1} \frac{N_{tank}^{N\_tank}}{(N_{tank}-1)!} \cdot \exp\left(-\frac{t \cdot N_{tank}}{t_m}\right) \tag{5}$$

2.6. Microbial community analysis of PVA gel beads in the pilot-scale AnMBBR

The microbial communities in the PVA gel beads of AnMBBR were identified using 16S rRNA gene amplicon sequencing (MiSeq). PVA gel beads were collected at the second and third ports of the reactor for each OLR with sterile equipment, and the samples were kept at −20 °C until further processing. The protocol for microbial community analysis is provided in the supplementary material (Text S1). The localization, biofilm structure, and morphology of microorganisms on the PVA gel beads at the end of AnMBBR operation were analyzed by SEM (JSM-IT300LV, Japan).

3. RESULTS AND DISCUSSION

3.1. Performance of the pilot-scale AnMBBR gels at different OLRs

The pilot-scale AnMBBR with PVA gel beads as media was operated at four different OLRs of 4.14 ± 1.33, 5.38 ± 1.22, 7.54 ± 1.53, and 12.50 ± 4.91 kg COD/m^3 day, which were applied by adjusting the flow rate to 350, 500, 750, and 1,000 L/d, respectively. The system was operated for a total period of 6 months (6 weeks for each OLR). The amount of time used was considered to be long enough to achieve reliable COD removal efficiencies for all OLRs.

3.1.1. COD removal efficiencies at different OLRs

The concentrations of total COD and sCOD at different OLRs are shown in Figure 2. The influent, which was real wastewater from the canned fish factory after primary treatment, had a total COD concentration of 3,204 ± 936 mg/L and sCOD concentration of 1,657 ± 397 mg/L. The COD fluctuations were likely due to changes in the raw materials, such as fish species (e.g., sardines, mackerels, and striped catfish).

Figure 3 shows the total COD removal efficiencies at four different OLRs, corresponding to flow rates of 350, 500, 750, and 1,000 L/d, which were 72.4 ± 6.4%, 65.5 ± 6.1%, 64.0 ± 4.7%, and 61.1 ± 7.8%, respectively. The sCOD removal efficiencies were higher than the total COD removal efficiencies, with values of 81.0 ± 12.4%, 76.8 ± 8.2%, 74.7 ± 6.2%, and 70.6 ± 12.4% at flow rates of 350, 500, 750, and 1,000 L/d, respectively. It should be noted that the total COD concentration includes the COD of the remaining suspended solids, which are typically more difficult to degrade than sCOD. In addition, the system was not designed to remove suspended solids. The suspended solid removal results are further discussed in Section 3.1.2.

Comparing the OLRs reveals that the COD removal efficiency decreased with increasing OLR, which is typical in anaerobic bioreactors (Torkian *et al.* 2003; Sánchez *et al.* 2005; Rashidi & Moghaddam 2021). In addition, similar patterns of COD removal efficiency were observed for all OLRs. In particular, immediately after an increase in the OLR, the COD removal efficiency decreased for a short period of time (approximately 5–15 days) before rebounding to a stable value. A possible explanation is that when the OLR was suddenly increased, microorganisms in the system could perceive it as a shock load, which can generally lower the COD removal efficiency. However, once microbial growth caught up with the increased OLR, the COD removal efficiency was likely to recover. Similar patterns of a sudden decrease followed by a rebound in the COD removal efficiency after an increase in the OLR were also previously observed in anaerobic bioreactors with PVA gel beads as media (Zhang *et al.* 2007, 2009; Wenjie *et al.* 2008). In addition, it is also possible that fluctuations in COD removal

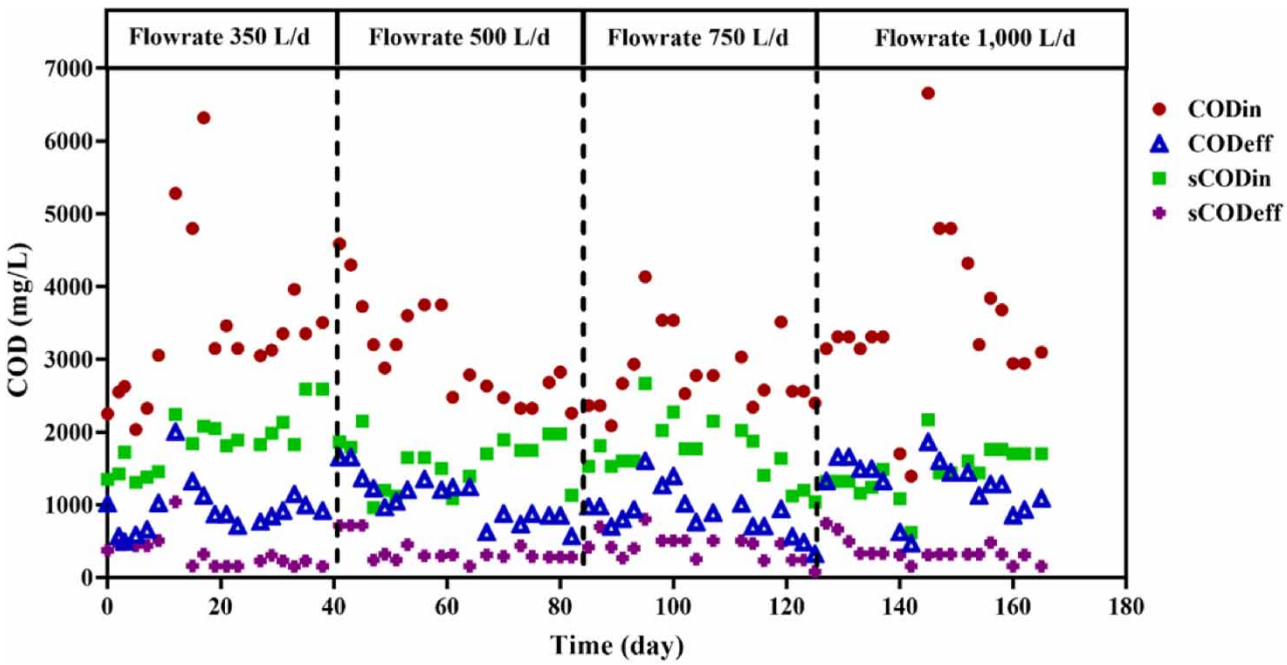


Figure 2 | Total COD and sCOD concentrations of the influent and effluent at different OLRs.

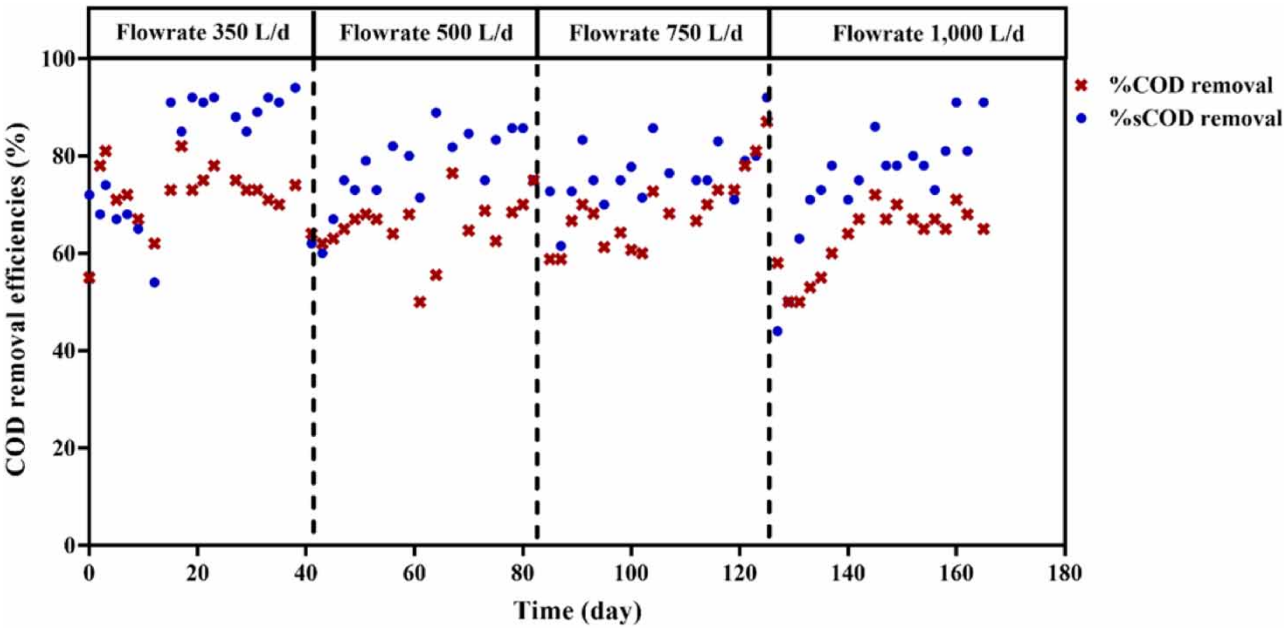


Figure 3 | Percent removal of total COD and sCOD at different OLRs.

efficiencies could be related in part to variations in the real wastewater compositions, e.g., COD concentration, C/N ratio, and fat, oil, and grease (FOG) contents, because raw materials (fish species) are often changed in the production of canned fish.

Overall, the total COD and sCOD removal efficiencies throughout the experiment were over 60 and 70%, respectively. The COD removal performances of the anaerobic bioreactors with PVA gel beads as media in this study and in previous studies are summarized in Table 1. Due to the differences in wastewater characteristics, reactor configuration, operating parameters, and environmental conditions, the results cannot be directly compared. Nevertheless, the COD removal efficiency achieved in this study appeared to be in a low range of what was observed in previous studies (69–95%).

The pilot-scale AnMBBR operated in this study consisted of six ports. Wastewater entered the reactor at the first port from the bottom, flowed upward, and was discharged at the highest port. The average COD and sCOD values at these ports at different OLRs are shown in Supplementary Figure S1. From the results, similar trends were observed in all OLR conditions, where the highest COD concentration was observed at the first port, and most of the COD and sCOD (>65 and >75%, respectively) were primarily removed within the second port. A possible explanation is that the first port was exposed to the highest COD concentration, resulting in the highest rate of COD removal compared to those of the other ports. Moreover, most of the PVA gel beads were located below the second port.

3.1.2. Monitoring parameters

The pH, ORP, VFAs, alkalinity, and TSS in the system were monitored regularly. The system was continuously operated with a pH of 7.60 $\pm$ 0.15 for the influent and 7.12 $\pm$ 0.04 for the effluent (Figure 4(a)). The pH values were in the range that is suitable for anaerobic bioreactors, i.e., 6.6–7.6 (Rittmann & McCarty 2001). Throughout the AnMBBR operation, the ORP values were -296 ± 52 mV for the influent and -385 ± 69 mV for the effluent (Figure 4(b)), suggesting that the AnMBBR could be maintained under anaerobic conditions throughout the total course of operation.

The VFA concentrations were 376 $\pm$ 120 mg/L as CH_3COOH and 418 $\pm$ 146 mg/L as CH_3COOH at the second and third sampling ports from the bottom of the reactor, respectively (Supplementary Figure S2(a)). At the beginning (flow rate of 350 L/d), the VFA concentration in the AnMBBR was quite high. Most likely, methanogenesis was not yet in equilibrium with fermentation activities at this initial stage of AnMBBR operation, resulting in VFA accumulation. However, after a certain period of AnMBBR operation, the VFA concentration decreased and remained constant at flow rates of 500, 750, and 1,000 L/d, indicating that methanogenesis and fermentation were in good equilibrium despite the increase in the OLR. Increasing the OLR did not appear to have an effect on the VFA concentration. The results were inconsistent with those observed in Wang *et al.* (2018b), who found that VFAs increased with the OLR. Nevertheless, it should be noted that

Table 1 | COD removal performances of anaerobic bioreactors in this study and previous studies using PVA gel beads as media under mesophilic conditions

Reference	Type of reactor	Type of wastewater	HRT (h)	Reactor volume (L)	Performance
Laboratory scale					
Zhang *et al.* (2007)	UASB	Corn steep liquor	12–48	7.5	COD removal efficiency of over 90% was maintained under continuous operation at an OLR up to 22.5 kg COD/m^3 day
Wenjie *et al.* (2008)	UASB	Corn steep liquor	10–48	7.5	COD removal efficiency was greater than 87% at an OLR of 22.5 kg COD/m^3 day
Zhang *et al.* (2009)	AFB	Corn steep liquor	6–10	3.9	COD removal efficiency at 91% was achieved at an OLR of 27.5 kg COD/m^3 day
Khanh *et al.* (2011)	UASB	Peptone–bonito extract	0.2–2.0	3.9	COD removal rate reached 28 kg COD/m^3 day at 35 °C
Jin *et al.* (2016)	EGSB	Ethylene glycol	–	3.9	COD removal efficiency reached higher than 95% with an OLR of 15 kg COD/m^3 day
Yue *et al.* (2016)	UASB	Ethylene glycol	8–14.4	7.5	COD removal efficiency reached higher than 90% with an OLR of 11 kg COD/m^3 day
Wang *et al.* (2018b)	UASB	Pharmaceutical wastewater	12–15	2.5	COD removal efficiency was in the range of 69–75% at an OLR of 7 kg COD/m^3 day
Pandey & Sarkar (2017)	AnPBR (2 stage)	Molasses	36, 60	13	COD removal efficiency was 89% at an OLR of 0.5 kg COD/m^3 day
Jeong *et al.* (2017)	AnCMBR	Domestic wastewater supplemented with food waste recycling wastewater	13	24	COD removal efficiency was 98.7% at an OLR of 2.95 kg COD/m^3 day
Pilot scale					
This study	AnMBBR	Fish canning industry	6.9	288	The sCOD removal efficiencies were above 70% for all OLRs (3.0–8.7 kg COD/m^3 day)

UASB, upflow anaerobic sludge blanket; AFB, anaerobic fluidized bed; EGSB, expanded granular sludge bed; AnPBR, anaerobic packed bed reactor; AnCMBR, anaerobic ceramic membrane bioreactor; AnMBBR, anaerobic moving-bed biofilm reactor.

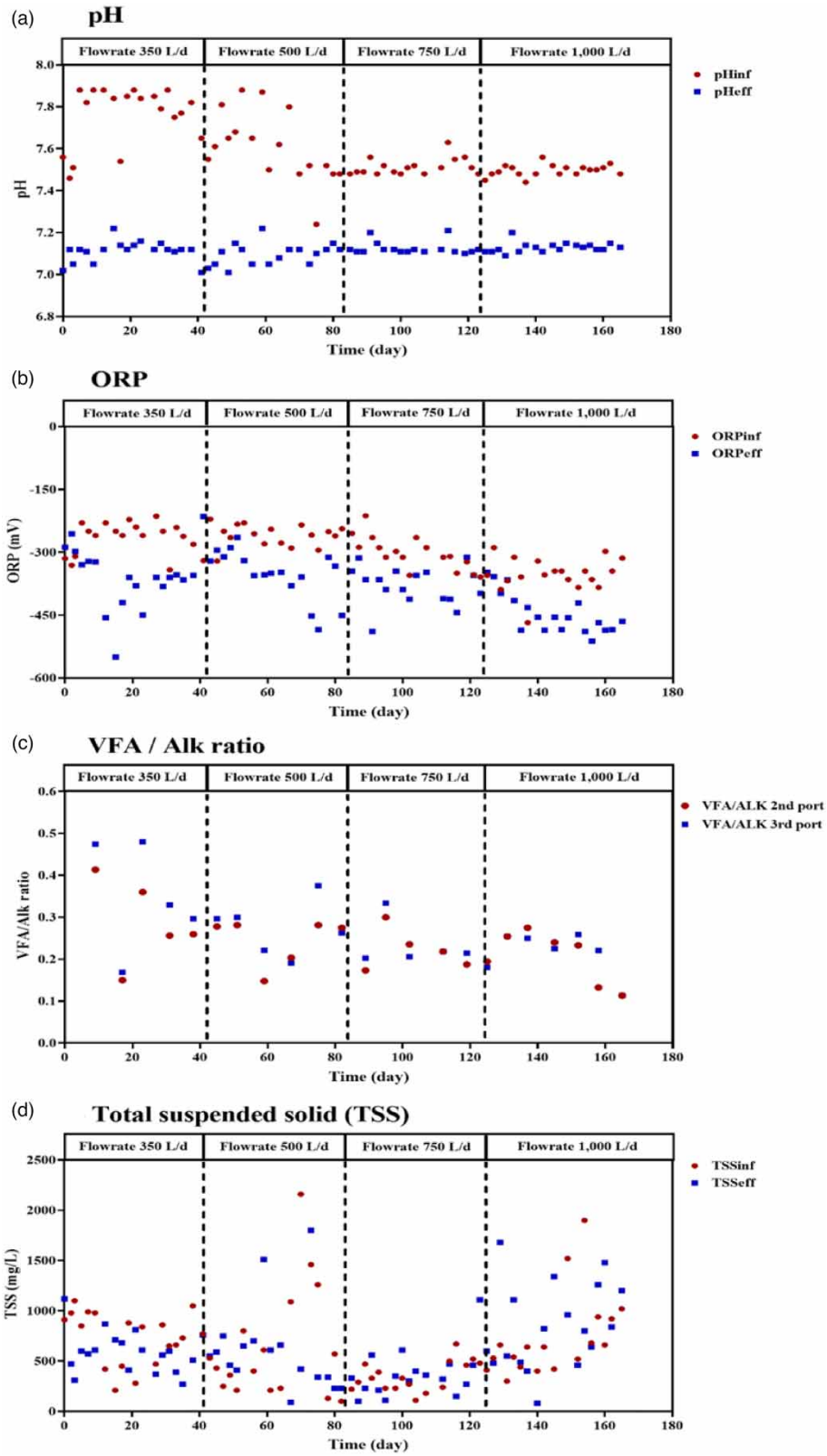


Figure 4 | Monitoring parameters for controlling the pilot-scale AnMBBR reactor, including the pH (a), ORP (b), VFA/alkalinity ratio (c), and TSS (d).

sufficient alkalinity was provided in this study with 2,000 mg/L as $CaCO_3$ in the influent, which was effective in controlling the pH and VFA accumulation. The alkalinity was 1,615 ± 630 mg/L as $CaCO_3$ and 1,626 ± 554 mg/L as $CaCO_3$ for the second and third ports of the reactor, respectively (Supplementary Figure S2(b)). The VFA/alkalinity ratios were in the range of 0.23 ± 0.08 and 0.26 ± 0.09 for the second and third ports of the reactor, respectively (Figure 4(c)), which were in the range of 0.1–0.3 recommended for the stable operation of anaerobic treatment processes (Padilla-Gasca *et al.* 2011).

Regarding suspended solids, the TSS concentrations were 657 ± 513 and 643 ± 483 mg/L in the influent and effluent, respectively (Figure 4(d)), with high fluctuations. The results suggest that AnMBBR with PVA gel was not effective in TSS removal. Moreover, since the TSS concentrations in the influent and the effluent were approximately the same, suspended microorganisms were unlikely to be maintained in this system, which was expected due to the high upflow velocity (1.5 m/h) in the reactor. In other words, the main microbial activities were likely to be from those retained on the PVA gel media rather than from suspended microorganisms.

Biogas production from the system was observed at flow rates of 350 and 750 L/d, with values of 313 ± 142 and 424 ± 265 L/d, respectively. The measured biogas production was less than the theoretical values of 556 and 1,012 L/d for flow rates of 350 and 750 L/d, respectively. However, it is unclear whether all total COD, which included suspended solids, was removed via biogas production. If we consider biogas production based on sCOD removal, the values were closer to the theoretical values, which were 303 and 662 L/d for flow rates of 350 and 750 L/d, respectively. Additionally, the components of the biogas collected near the end of operation were methane gas (82.45 ± 1.91%), carbon dioxide (12.19 ± 2.40%), and other (5.35 ± 2.63%). The percentages of methane found in this study were rather high compared to those reported in other research (Chaikasem *et al.* 2014; Jeong *et al.* 2017).

3.2. RTD in the pilot-scale AnMBBR with PVA gel

Table 2 shows the theoretical and mean residence times of the AnMBBR at flow rates of 350 and 1,000 L/d. The results indicate that both the theoretical and mean residence times were higher when a lower flow rate was used. However, the mean residence time (t_m) values for both flow rates were significantly higher than the theoretical residence times, indicating very strong external recirculation inside the AnMBBR. This strong recirculation was consistent with the AnMBBR operating conditions, where the flow rate of effluent recirculation was much higher than the inlet flow rate, leading to multiple passes of the fluid inside the AnMBBR. The flow can be characterized according to the ratio between the mean and theoretical residence times (t_m/t_{theo}), where a ratio in the range of 1.7–1.9 indicates 2-pass or 3-pass external recirculation flow for the AnMBBR. In detail, the tank-in-series model and the compartment model were further used to analyze the flow pattern of the AnMBBR.

Figure 5(a) and 5(b) shows $E(t)$ (the E-curve) as a function of time for flow rates of 350 and 1,000 L/d, respectively. Slightly positive skewness curves were achieved for both flow rates, with stronger positive skewness for the lower flow rate. This result occurred because the flow pattern was closer to the ideal plug flow regime at a higher flow rate than at a lower flow rate (Peña *et al.* 2006). A strong early peak was observed when the time was less than 20 min for both flow rates. According to the compartment model, this early peak indicates short-circuit flow inside the column. Moreover, this short-circuit flow was stronger when a flow rate of 1,000 L/d was used, as the higher flow rate resulted in a higher velocity of the fluid flowing inside the column, which eventually promoted the short-circuit flow regime. Notably, this short-circuit flow of the UASB was only observed when an external recirculation system was present since the velocity inside the column was significantly higher due to recirculation (Bayoumi 2007). Without recirculation, short circuits were not observed (Escudie *et al.* 2011; Montiel *et al.* 2019).

The tank-in-series model was used to analyze the recirculation of the AnMBBR. Nonlinear regression was performed by comparing the dimensionless E-curve from the experiment with the tank-in-series model to obtain the optimum number of

Table 2 | Theoretical residence time, mean residence time, and tank-in-series model parameters of the AnMBBR

				Tank-in-series model		
Flow rate (L/d)	**t_{theo} (min)**	**Mean residence time, t_m (min)**	**t_m/t_{theo}**	**N_{tank}**	**Residence time per tank (min)**	**t_{tank}/t_{theo}**
350	1,185	2,111	1.78	3	880	0.74
1,000	414	804	1.94	3	338	0.82

t_{tank}, residence time per tank.

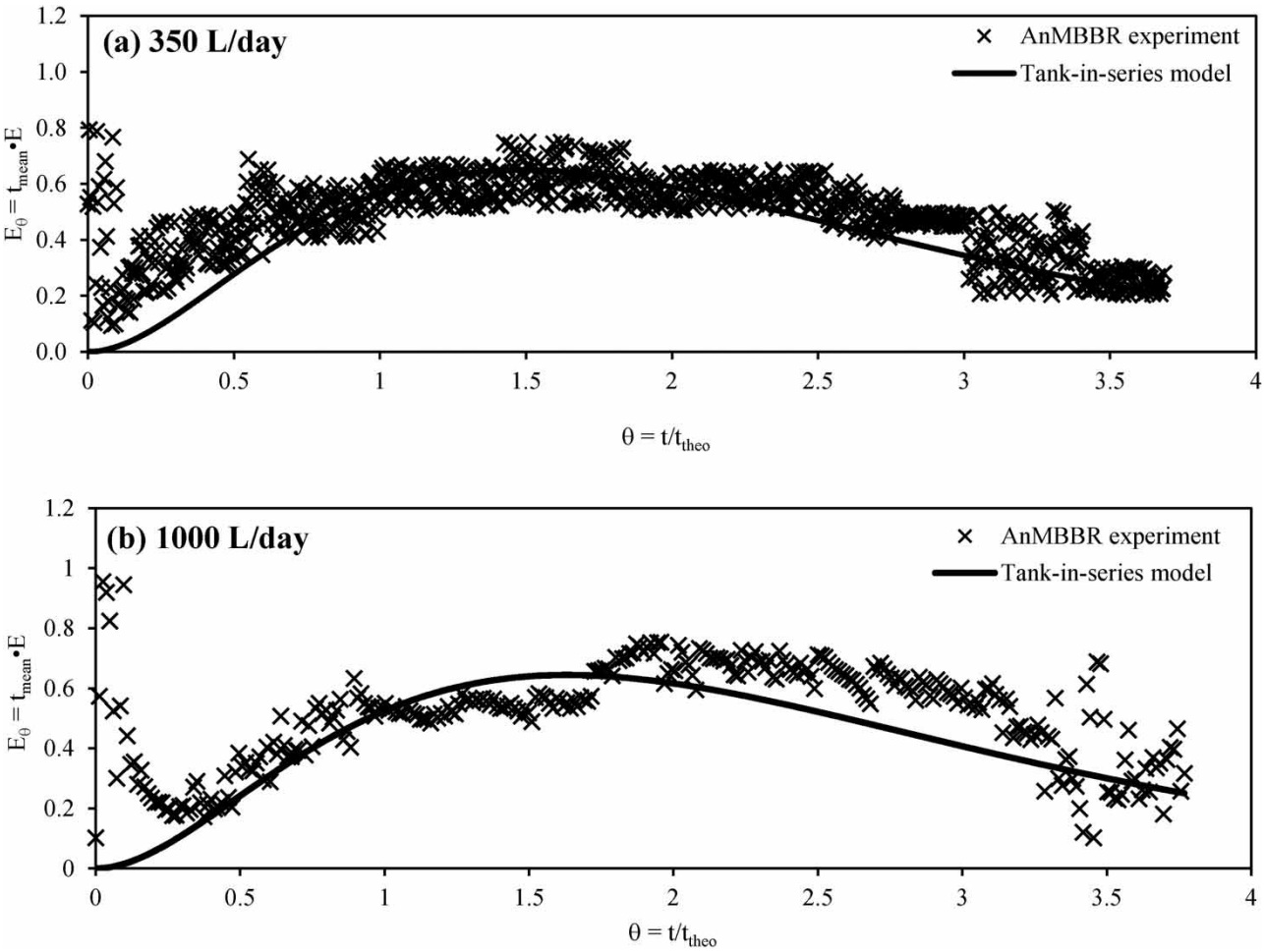


Figure 5 | Dimensionless E curves of the pilot-scale AnMBBR with PVA gel and the tank-in-series model for different flow rates: (a) 350 L/d and (b) 1,000 L/d.

tanks-in-series and the residence time of each tank. The smallest deviation between the tank-in-series model and the experimental results was achieved when the number of tanks-in-series (N_{tank}) was equal to 3 and the residence time of each tank was 880 and 338 min for flow rates of 350 and 1,000 L/d, respectively. The results of the tank-in-series model, expressed in the dimensionless E-curve, are plotted in Figure 5. When excluding the short-circuit peak that could not be determined by the tank-in-series model, the model results had good agreement with the experimental results, with an average error of less than 20%. This result supported the multipass presumption mentioned earlier and indicated that the fluid might have a 3-pass flow pattern through the AnMBBR.

3.3. Microbial community of PVA gel beads in the pilot-scale AnMBBR

3.3.1. 16s rRNA gene amplicon sequencing (MiSeq, Illumina)

The microbial communities in the seed sludge and on the PVA gel beads after being exposed to different OLRs were analyzed by 16S rRNA gene amplicon sequencing (MiSeq, Illumina), as shown at the phylum level in Figure 6. The results clearly suggest a shift in microbial communities after AnMBBR operation compared to the seed sludge despite the same substrate being provided. Such a shift was expected since the operating conditions of the UASB from which the seed sludge was taken and the pilot-scale AnMBBR were totally different. For example, microorganisms in UASBs undergo selective pressure to form granules, whereas those in the pilot-scale AnMBBR were attached within the PVA gel beads.

However, the microbial communities on the PVA gel beads after operating at different OLRs were quite similar, and those of the second and third ports were apparently the same. A possible explanation is that although the OLRs were different, the

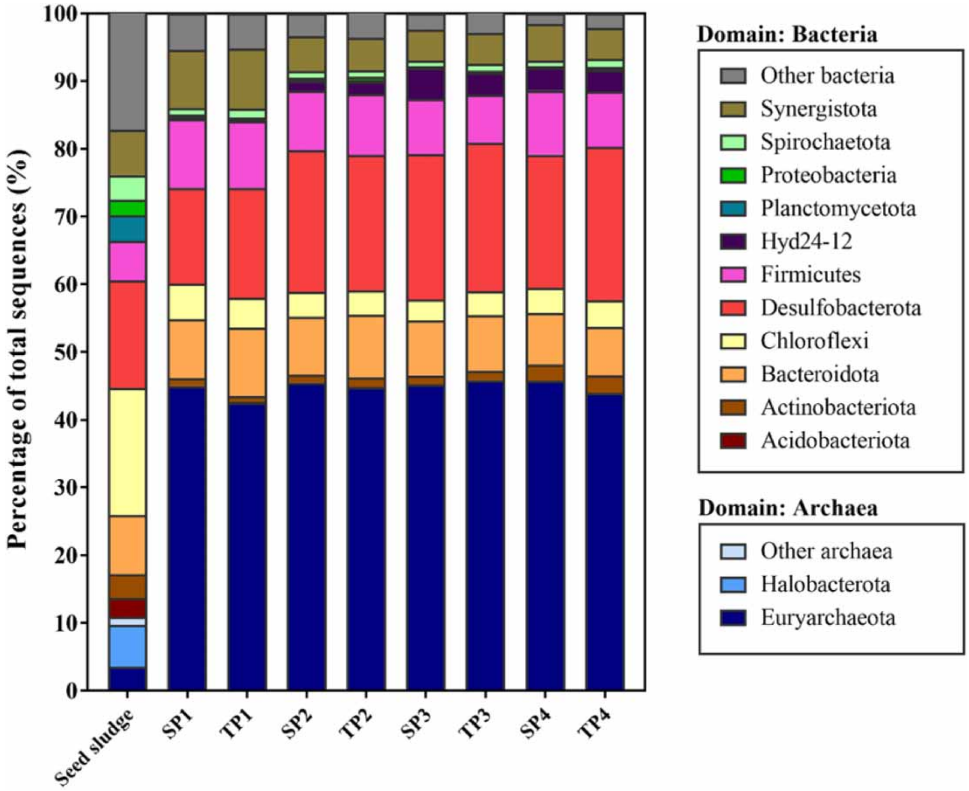


Figure 6 | Microbial communities in the seed sludge and on PVA gel beads in the pilot-scale AnMBBR at the phylum level (SP, second port at 0.75 m in height; TP, third port at 1.25 m in height; different OLRs: 1 = 4.14 kg COD/m^3 day; 2 = 5.38 kg COD/m^3 day; 3 = 7.54 kg COD/m^3 day; and 4 = 4.14 kg COD/m^3 day).

substrate concentrations in the pilot-scale AnMBBR remained approximately the same. Substrate concentrations are generally considered to be one of the key factors influencing microbial communities in anaerobic bioreactors (Pasalari *et al.* 2021).

The archaeal and bacterial phyla with the five highest relative abundances in the seed sludge and on the PVA gel beads were Euryarchaeota (3.5–45.9% of total sequences), Bacteroidota (7.1–10.0% of total sequences), Desulfobacterota (14.1–22.7% of total sequences), Firmicutes (5.9–10.15% of total sequences), and Synergistota (4.5–8.9% of total sequences).

Over a hundred genera were found in all of the samples, and these genera can be divided into three main groups according to their metabolic functions: (1) hydrolytic/acidogenic bacteria, (2) acetogenic bacteria, and (3) methanogens. The 25 most relatively abundant genera and families are summarized in Figure 7. Although four different OLRs were applied in the pilot-scale AnMBBR, the predominant microorganisms appeared to be rather similar.

Hydrolytic/acidogenic bacteria, such as *Paraeggerthella* spp., unclassified genera in the order Bacteroidales, SJA-15, *Syntrophomonas*, SJA-88, and W22, were observed in the pilot-scale AnMBBR, and they were likely to be responsible for hydrolysis and acidogenesis in the system, i.e., the degradation of organic compounds into VFAs (Wang *et al.* 2018a). Regarding acetogenesis, the dominant acetogenic bacteria in the pilot-scale AnMBBR included *Desulfomicrobium* spp., unclassified genera in the family Syntrophaceae, *Syntrophobacter* spp., unclassified genera in the family Syntrophorhabdaceae, *Syntrophomonas* spp., and HA73 spp. These acetogenic bacteria degraded VFAs to intermediate substances, such as acetate, hydrogen, and carbon dioxide for methanogens (Amani *et al.* 2010).

Regarding methanogenesis, two major genera of methanogens, *Methanosaeta* and *Methanobacterium*, were observed. *Methanosaeta* was abundantly detected in the pilot-scale AnMBBR after operating at different OLRs, with the highest relative abundances of 24.0–28.9% of the total sequences. *Methanosaeta* spp. are acetoclastic methanogens that solely utilize acetate to produce methane (Janssen 2003). The presence of *Methanosaeta* spp., a slow-growing methanogen, in the AnMBBR could be explained by their immobilization on PVA gel beads, which may allow them to have a longer residence time in the system to suffice their slow growth rates (Khanh *et al.* 2011). In addition, *Methanobacterium* spp., a hydrogenotrophic methanogen, was also found in the pilot-scale AnMBBR, accounting for 7.7–14.9% of the total sequences. Similarly, *Methanobacterium*

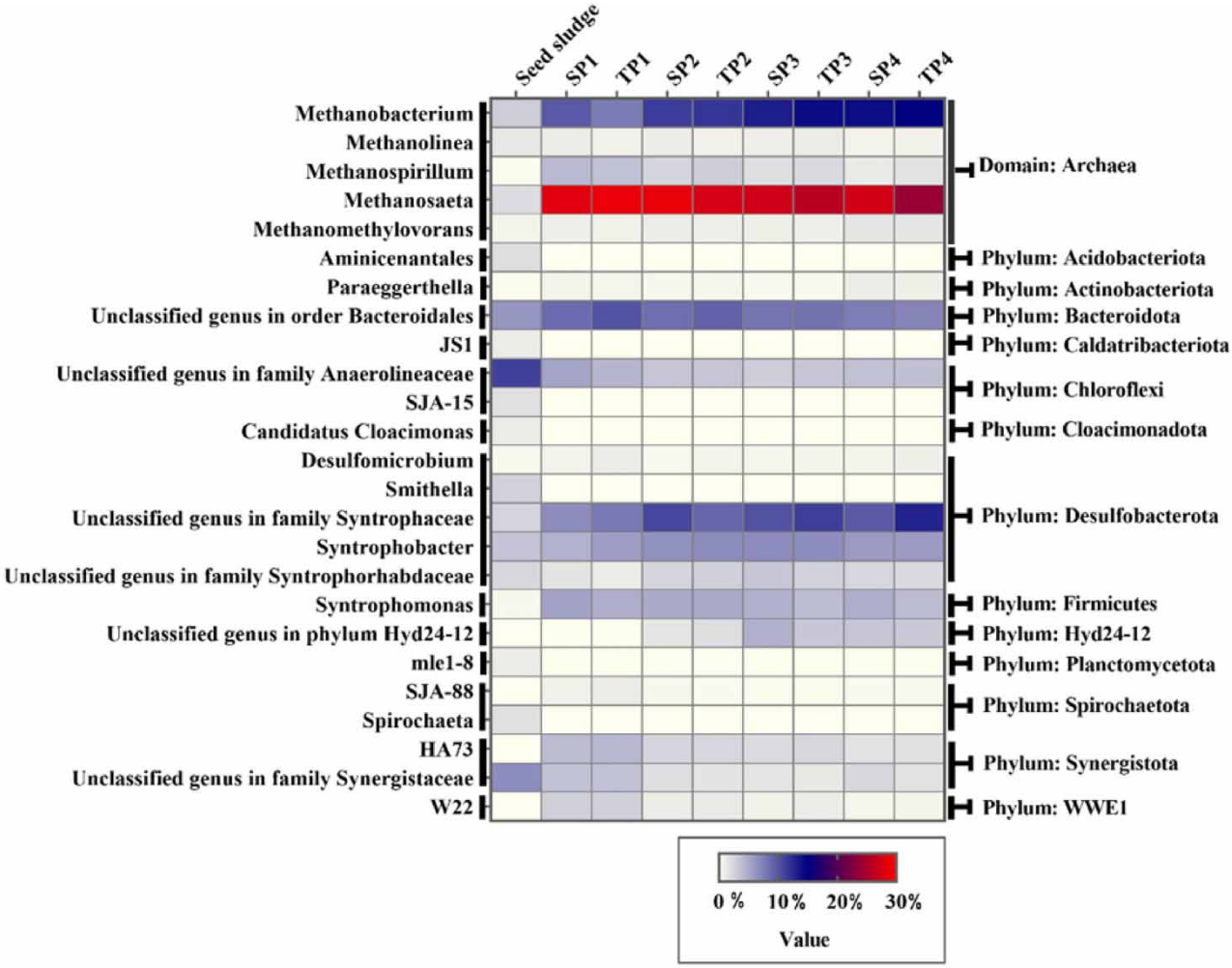


Figure 7 | The top 25 most abundant genera found in the seed sludge and on PVA gel beads in the pilot-scale AnMBBR (SP, second port at 0.75 m in height; TP, third port at 1.25 m in height; different OLRs: 1 = 4.14 kg COD/m^3 day; 2 = 5.38 kg COD/m^3 day; 3 = 7.54 kg COD/m^3 day; and 4 = 4.14 kg COD/m^3 day).

spp. and *Methanosaeta* spp. were observed in PVA gel beads in a UASB reactor treating synthetic wastewater containing peptone and bonito extract, but *Methanobacterium* spp. were present at a higher relative abundance than *Methanosaeta* spp. (Khanh *et al.* 2011). *Methanobacterium* spp. was also detected along with *Methanosarcina* spp. in a methanogenic reactor in two-stage anaerobic packed bed reactors (Pandey & Sarkar 2019).

3.3.2. SEM

PVA gel beads that had been used in the pilot-scale AnMBBR for 6 months were collected to investigate the localization of microorganisms using SEM. The virgin PVA gel beads had an average diameter of 2.150 ± 0.405 mm. In contrast, the average diameter of the PVA gel beads after usage in the pilot-scale AnMBBR increased to 4.175 ± 0.017 mm (Supplementary Figure S3). It should be noted that the PVA gels used for SEM analysis were rinsed with water during sample preparation; therefore, the outer surfaces were relatively smooth. The results suggest that microorganisms were successfully cultivated within the PVA gels, and the average diameter of the PVA gels increased by approximately 2 mm.

Most of the microorganisms were abundant near the outer layer (1.994 ± 0.266 mm) of the PVA gel beads (Supplementary Figure S4). The pores in the PVA gel beads could be separated into two zones, Zone A (center) and Zone B (outer layer), that had different pore sizes (Supplementary Figure S5). According to Pandey & Sarkar (2017), the pore sizes in Zone A were in the range of 10–30 μm. In the present study, Zone A had a pore size of 24 ± 7 μm, whereas Zone B had a smaller pore size of

1.2 ± 0.5 μm. Despite the large pore sizes, fewer microorganisms were observed in Zone A. A similar observation was made with immobilized anammox sludge on PVA gel beads, where bacteria were observed only in the outer layer (Zone B) of the PVA gel beads (Hoa *et al.* 2006). This phenomenon was explained by the dense layer of microorganisms formed in the outer layer (Zone B) hindering the penetration of microorganisms into the center of the PVA gel beads (Zone A) (Hoa *et al.* 2006). Another possible explanation is that substrates may be consumed through the outer layer of the beads, thereby not being available in the center of the PVA gel beads (Hoa *et al.* 2006). However, the results were opposite to those of Zhang *et al.* (2009), who found more microorganisms in Zone A.

Figure 8 shows an SEM image of PVA gel beads at 5,000× magnification, which revealed the presence of microorganisms with rod shapes with flat ends (A) and spherical shapes (B). The coexistence of these microorganisms was also observed in PVA gel beads in a different anaerobic system used for fermentative hydrogen production (Yin *et al.* 2018). Microorganisms with rod shapes with flat ends were the most abundant in the PVA gel beads in our pilot-scale AnMBBR. These microorganisms were likely to be *Methanosaeta* spp. according to their morphology. The results are in agreement with a previous study by Jin *et al.* (2016) that reported the predominance of *Methanosaeta* spp. in an expanded granular sludge blanket (EGSB) reactor treating high-strength ethylene glycol wastewater with PVA gel beads as media. The results are consistent with those from 16S rRNA gene amplicon sequencing (MiSeq) that found *Methanosaeta* spp. at the highest relative abundance in the pilot-scale AnMBBR.

It should be noted that the localization of microorganisms in conventional UASB granules and PVA gel beads was different. In the UASB granules, methanogens were located in the centers of the granules, while fermentative bacteria were localized on the outside (Fang 2000). In contrast, most of the microorganisms in the PVA gel beads were found near the outer layers, as shown by the SEM results in this study. These differences in microbial localization could lead to differences in the transfer of intermediate substrates, e.g., VFAs, H_2, and CO_2, in AnMBBRs and conventional UASBs, which could affect the balance of methanogenesis and fermentation activities and microbial communities. This finding could help explain the differences in microbial communities in the AnMBBR compared to the seed sludge obtained from the UASB (Figure 6), even though the same substrate was provided.

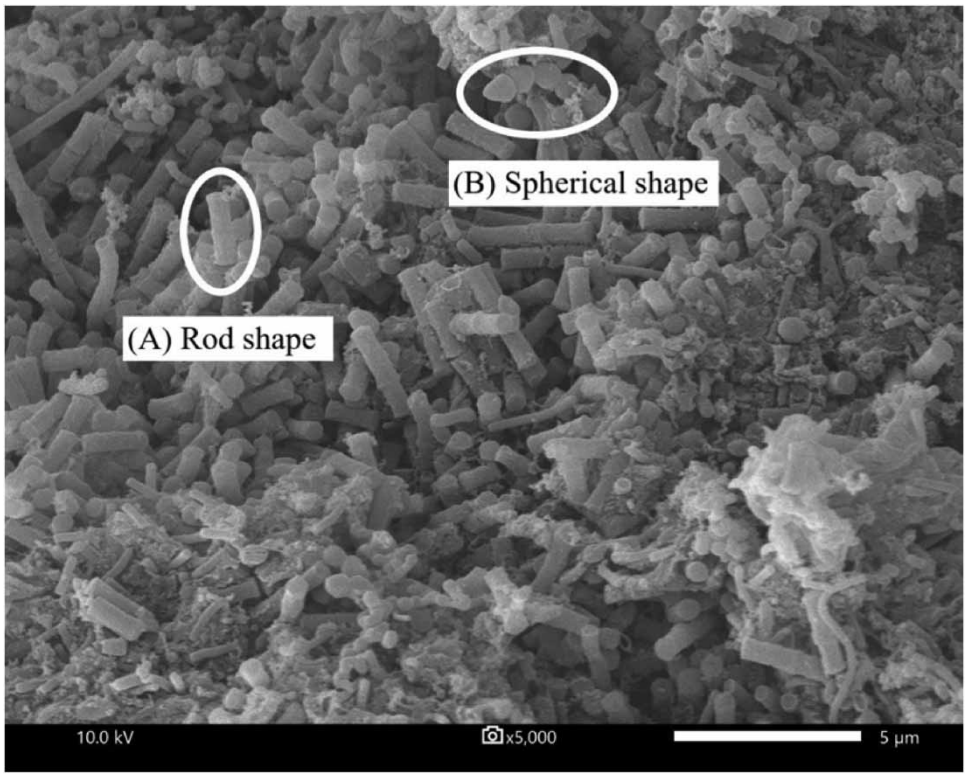


Figure 8 | Microorganisms in the PVA gel beads at 5,000× magnification.

3.4. Engineering implications and limitations

In this study, the application of an AnMBBR using PVA gel beads as media was successfully demonstrated at the pilot scale using wastewater from a fish canning factory. The results show that the overall COD removal efficiency of the pilot-scale AnMBBR was above 60%, whereas the average sCOD removal efficiency was above 70% for all OLRs (3.0, 4.3, 6.5, and 8.7 kg COD/m^3 day). In addition, the pH, alkalinity, VFAs, and ORP were stable and in ranges suitable for anaerobic reactors. The design values used in this study, e.g., an OLR of 3.0–8.7 kg COD/m^3 day and an upflow velocity of 1.5 m/h, can be adopted as design criteria for full-scale AnMBBRs using PVA gel beads as media for similar wastewaters. In addition, pretreatment processes for TSS removal prior to AnMBBRs are recommended since the systems do not exhibit effective TSS removal. Aerobic wastewater treatment systems are also required as post-treatment processes to achieve high-quality effluents regarding COD concentrations.

Nevertheless, it should be noted that this study has certain limitations. First, the upflow velocity was not optimized. Since the upflow velocity was not varied in this study, it is possible that higher or lower upflow velocities may be used, which could result in different system performance. Second, fat, oil, and grease (FOG) contents in wastewater are another factor that could influence the attachment of microorganisms in PVA gel beads, which was not investigated in this study. Short-circuit flow is another concern in the AnMBBR, especially at high flow rates, suggesting that the reactor design, e.g., flow distribution system, should be improved in the future. In addition, the durability of the PVA gel beads and the accumulation of microorganisms over long-term operation were not investigated. The limitations of this study require further studies to achieve the full potential of AnMBBRs.

4. CONCLUSION

This study demonstrated for the first time the successful operation of a pilot-scale AnMBBR using PVA gel beads as media for the treatment of wastewater from a fish canning factory. For all OLRs (3.0, 4.3, 6.5, and 8.7 kg COD/m^3 day), the average total COD removal efficiency of the pilot-scale AnMBBR was above 60%, whereas the average sCOD removal efficiency was above 70%. The RTD results suggest that the fluid had a 3-pass flow pattern through the pilot-scale AnMBBR. The microorganisms were primarily located inside the PVA gel beads near the outer surfaces. The results from 16S rRNA gene amplicon sequencing (MiSeq) and SEM analysis revealed that *Methanosaeta* spp. were the predominant microorganisms in the pilot-scale AnMBBR. OLRs of 3–8.7 kg COD/m^3 day are recommended as design criteria for full-scale AnMBBRs with PVA gel beads as media for similar wastewaters along with pretreatment processes for TSS removal and post-treatment processes for further COD removal. Future research on the optimization of the upflow velocity, the effects of FOG on the attachment of microorganisms, designs of flow distribution systems in AnMBBRs, and durability of PVA gel beads is recommended.

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CONFLICTS OF INTEREST STATEMENT

This research was funded in part by Kuraray Co., Ltd, Japan, a company that manufactures PVA gel beads. The pilot-scale AnMBBR and PVA gel beads were also provided by Kuraray Co., Ltd, Japan.

DATA AVAILABILITY STATEMENT

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

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