

Article

## Effect of Mixing Driven by Siphon Flow: Parallel Experiments Using the Anaerobic Reactors with Different Mixing Modes

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Received: 8 June 2013; in revised form: 25 July 2013/ Accepted: 5 August 2013 /

Published: 19 August 2013

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**Abstract:** The effect of mixing by siphon flow on anaerobic digestion, sludge distribution and microbial community were examined in parallel experiments using a siphon-mixed reactor (SMR), an unmixed reactor (UMR) and a continuously mixed reactor (CMR). The SMR performed well without the accumulation of fatty acids under COD loading rates varying from 3 to 18 kg/m<sup>3</sup>/day, while the UMR was totally acidified when the loading rate increased to 10 kg/m<sup>3</sup>/day. The methane yield of the SMR was at least 10% higher than that of the UMR, and comparable to that of the CMR. Furthermore, the SMR was found to markedly improve the dispersion of solids and reduce deposit formation compared to the UMR. Besides, during stable operation, the fatty acids level in the effluent of the SMR and UMR was lower than that in the CMR, and the archaeal community structure of the SMR was similar to that of the UMR.

**Keywords:** anaerobic digestion; siphon mixed reactor; mixing mode; sludge distribution; microbial community

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## 1. Introduction

Small-scale anaerobic digesters have been put to use mainly in developing countries [1], and there has been a recent significant increase in the number of small-scale digesters in operation in Asian countries [2,3]. At present, a very large number of small-scale digesters are in operation in China and India in particular, with over 30 million in the former [2], and 4 million in the latter [3]. These biogas plants are used to provide fuel and fertilizer instead of the firewood and animal dung typically used in poor rural areas for these purposes. Usually, biogas is used for cooking and lighting in households. Since cooking and lighting energy constitute most of the total energy consumed in rural households in developing countries [4], biogas can meet a large part of the household energy demands. Furthermore, the use of digesters brings other benefits: living conditions are significantly raised thanks to these new kitchens which use biogas instead of the old firewood, coal and dried stalk-fired kitchens [5]. Since the purpose of this type of digester is to create energy for poor households in rural areas, the cost of construction and operation must be very low. Typical digesters are unstirred and unheated systems, and as such, they are quite inefficient. Small-scale digesters require a very long hydraulic retention time (HRT), usually in excess of 60 days, to substantially degrade organic materials. Although many digesters have been built, further research and awareness is still needed, and it is crucial that researchers and engineers take the local and economical considerations into account when designing efficient small-scale digesters [2].

The efficiency of anaerobic digestion is affected by various factors, such as feeding patterns, sludge retention time, temperature, mixing and pH. Since mixing has been shown to have a large positive impact on digestion efficiency, most commercial large-scale digesters have a continuous mixing system. The effect of mixing on anaerobic digestion has been investigated from several perspectives [6–9]. The most notable positive effects of mixing are: (1) the dispersion of the substrate for better contact with microorganisms; (2) making the sludge temperature uniform and (3) the reduction of scum and deposits formation. It has been reported that efficient mixing promotes methane production and the destruction of volatile solids (VS), and that that digesters without mixing do not as well [8,10,11]. Further, studies focused on large-scale sewage sludge digesters have shown that inadequate mixing causes solid deposition and scum formation, resulting in short-circuiting [11]. The same is true for small-scale digesters since most do not have mechanical devices to mix and uniformly feed materials. Conventional small-scale digesters have been found to have a large amount of dead space [12], which leads to reduced actual HRTs compared to the calculated HRTs and, consequently, poor performance [1]. The dead space created is thought to be due to accumulated deposits, a scum layer and the digester configuration itself [12]. In this context, there is a clear need for a simple inexpensive small-scale digester design capable of mixing the materials fed.

A simple powerless mixing system using a siphon flow, which enables the successful operation of anaerobic digestion [13], is thought to have much potential for use in small-scale digesters. A fluid dynamics analysis has shown a significant improvement in sludge mixing when the reactor is mixed by the siphon flow [14]. As such, this mixing system is expected to enhance digestion performance and avoid stratification, without creating excessive costs. However, the previous studies [13,14] did not adequately assess the advantages of the siphon mixed reactor (SMR) over the standard unmixed reactor. In addition, there is a need to examine a difference between the SMR and the continuously

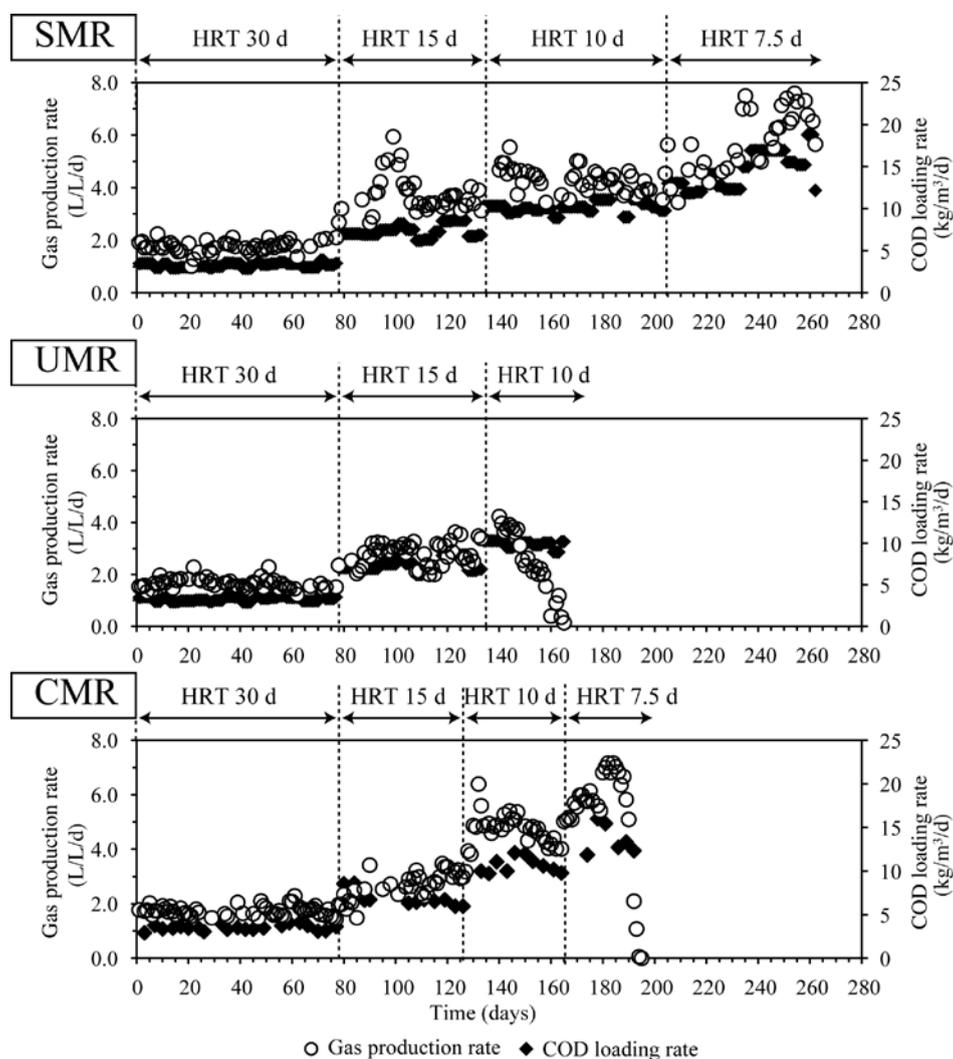
mixed reactor (CMR), in order to determine whether siphon mixing is actually capable of serving as a substitute for the commonly used digesters with continuous mechanical mixing. The present study is aimed to examine the effect of mixing by siphon flow on: (1) the digestion performance, (2) the sludge distribution characteristics and (3) the microbial community structure in parallel experiments using an SMR, an unmixed reactor and a CMR. The three types of reactors were operated semi-continuously with different mixing modes: siphon mixing, unmixing and continuous mixing by impellers. The advantages and limitations of siphon mixing were determined.

## 2. Results and Discussion

### 2.1. Acceptable Organic Loading Rates and Hydraulic Retention Time

Figure 1 illustrates the time course of the HRT of the feedstocks, the organic loading rate (OLR) ( $\text{kg-COD}/\text{m}^3/\text{day}$ ) and the gas production rate per reactor volume ( $\text{L}/\text{L}/\text{day}$ ). The graphs show the operation period after an approximately 3 months start-up at a HRT of 60 days (data not shown). The HRT was gradually shortened from 30 days (Phase 1) to 15 days (Phase 2), 10 days (Phase 3) and 7.5 days (Phase 4) by changing the on-off interval of the timers for the feed pumps.

**Figure 1.** Time course of HRT, OLR and the biogas production rate.



The OLR ranged from 2.9 to 3.8 kg-COD/m<sup>3</sup>/day (Phase 1); from 6.9 to 8.7 kg-COD/m<sup>3</sup>/day (Phase 2); from 8.9 to 12.6 kg-COD/m<sup>3</sup>/day (Phase 3) and from 11.9 to 18.2 kg-COD/m<sup>3</sup>/day (Phase 4), respectively. Since gas production rate of the CMR became stable earlier than the SMR and UMR, the duration of each phase were relatively shortened in the CMR. During Phases 1 and 2, all the three reactors maintained active gas production, with the gas production rates increasing with shorter HRTs. The frequency of siphon mixing depends on biogas production rate in the C1 [13]. In this case, mixing occurred approximately five times (Phase 1) and 10 times (Phase 2) per day in the SMR. The pH in the effluent sludge was kept within the range between 7.0 and 8.0 in each reactor throughout Phases 1 and 2. The VFA concentrations in the effluent sludge ranged from almost 0 to 94 mg/L as acetate (SMR); from almost 0 to 89 mg/L as acetate (UMR); from 42 to 460 mg/L as acetate (CMR). The CMR showed the highest level of VFA among the three reactors. This is possibly due to one of the negative effects of continuous stirring: the disruption of microbial flocs and obstruction of VFA degradation by symbiotic microflora [6,15]. It can be assumed that the unstirred UMR and intermittently stirred SMR had less risk of such floc disruption. It has been reported that intermittent stirring reduces the VFA level [6]. Furthermore, compared with the CMR, the multi-compartmental structure of the SMR and UMR appears to help to avoid short-circuiting, allowing insufficiently digested sludge to pour out. During Phases 1 and 2, the alkalinity levels in the effluent sludge from each reactor were above 3750 mg/L as CaCO<sub>3</sub>, therefore the VFA to alkalinity ratios (VFA/ALK) in the effluent sludge were maintained at a level below 0.09. The VFA/ALK is commonly used to evaluate the stability of anaerobic processes [16], and the process can be considered unstable if the VFA/ALK value exceeds 0.4. From these results, it can be concluded that all three reactors achieved successful operations during Phases 1 and 2.

During Phase 3, the UMR showed a different trend from that of the other two reactors. As soon as the HRT was shortened from 15 days to 10 days, the gas production rate of the UMR gradually decreased, and finally, the gas production almost completely stopped (Figure 1). At this time, the sludge pH of C1 and C2 had fallen below 4.0, and that of C3 was also relatively low level, below 7.0. The VFA concentrations were more than 6000 mg/L in the form of acetate in compartments C1, C2 and C3. This clearly indicates that the gas stop of the UMR was due to acidification in the whole reactor. The pH level tended to rise, and the VFA concentration tended to decrease with distance from the inlet. As described above, the sludge pH of C1 had already dropped to around 4 during Phase 2. The acidification appeared to spread from the C1 throughout the entire the reactor with the stream. This type of VFA and pH pattern has often been observed in plug flow reactors [17–19]. The feedstock is firstly hydrolyzed and fermented to VFA by acidogenic bacteria, and is converted into acetate and H<sub>2</sub> by acetogenic bacteria, and is finally fermented to methane by methanogenic archaea. Hence, acidogenesis as a first-stage reaction tends to be relatively more active in the front part close to the inlet. In contrast to the UMR, the SMR and CMR maintained active methane production during Phase 3. The VFA concentrations in the effluent were kept at a low level below 300 mg/L as acetate, and the VFA/ALK was below 0.07 in the SMR and CMR. These results strongly suggest that siphon mixing resulted in an expansion of the acceptable ranges of OLR and HRT.

Subsequently, Phase 4 operation was characterized by difficulties in the SMR and CMR. Two weeks after the HRT was shortened to 7.5 days, the VFA concentration in the CMR began to increase. The gas production rate decreased in accordance with the increase in VFA. When the VFA concentration reached 8000 mg/L as acetate and the pH dropped to 5.1, the gas production of the CMR almost stopped. The maximum OLR for wet anaerobic digestion of food waste was investigated by Nagao [20], who reported that the theoretical maximum OLR of the mesophilic CMR was 17.0 kg-COD/m<sup>3</sup>/day (10.5 kg-VS/m<sup>3</sup>/day) under SRT 60 days and HRT 8 days. In the case of this study, the CMR began unstable operation at an OLR of 15.5–16.0 kg-COD/m<sup>3</sup>/day, close to the maximum OLR reported by Nagao. As such, it is likely that the CMR was disrupted by overloading during Phase 4.

As such, the SMR is the only reactor that continued active biogas production throughout the experiment. However, there was another problem with the SMR during Phase 4. As soon as the HRT was shortened to 7.5 days, the SMR experienced a significant increase in floating scum layer in the upper part of the compartments of C2 and C3. There was no such layer in the reactor during Phases 1 and 2. Scum began to appear late in the Phase 3 period. The depth of the scum plus the sludge during Phase 4 was typically 45 cm, whereas it was about 35–37 cm during Phases 1 and 2, implying the scum layer on the sludge surface was approximately 10 cm thick during Phase 4. This result was quite different from that obtained in our previous study [13], which involved the use of an artificial food waste feedstock. In this previous study, no scum formation was observed in the SMR during the OLR of 5–10 kg-COD/m<sup>3</sup>/day. The scum formation potential is likely to depend on the change of sludge characteristics, and in this case, due to an increase in the OLR level. There is a report that scum forming potential in an anaerobic digester is correlated with HRT shortening [21]. The HRT shortening resulted in an increase in biogas production, and a decrease in the degradation of organic matter such as proteins and lipids in the anaerobic sludge. The former can promote a flotation of organic materials, potentially forming a scum, and the latter increases the amount of organic materials which surface. Our finding, which was characterized by the increase in scum formation depending on the HRT shortening, seems to be consistent with the results reported by Halalsheha [21]. On the other hand, the frequency of siphon mixing increased according to the increase in OLR. Although mixing occurred only five times per day during Phase 1, it increased to about 15–20 times per day during Phase 4. It is well known that mixing reduces scum formation, but in this case, the mixing in the SMR does not seem to have been sufficient to avoid scum formation under the very high OLR. The scum taken from the upper part of the SMR was analyzed. The COD of the scum was 88.2 g/L, which was 200%–300% higher than the underlying sludge. This suggests that a significant part of organic matter fed as substrate accumulated in the upper part of the reactor. Furthermore, a slight increase in the sedimented solids was observed at the front part of the SMR after the HRT was shortened to 7.5 days. Therefore, the mixing occurred in the SMR seems to be insufficient for solid dispersion. However, this is possibly the reason why the SMR was the only reactor avoided serious acidification during Phase 4. The 7.5 days of HRT is too short for continuous cultivation of methanogens because the growth rates of methanogens are generally slow, and finally, a short HRT would wash out large amount of the methanogens. Perhaps decrease in methanogens population led to the VFA accumulation in the CMR during Phase 4. On the other hand, relatively poor mixing likely enabled the SMR to retain larger amount of methanogens in the reactor, which resulted in higher methanogenic activity.

## 2.2. COD Reduction and Methane Conversion

Table 1 summarizes the digestion performance of each reactor at different HRT conditions. The data in the table are the average values during the period after a time equivalent to each HRT. The  $Red_{COD}$  decreased with shortened HRT in all the reactors. Under the same HRT, the  $Red_{COD}$  levels of the SMR and UMR were a little higher than those of the CMR. On average, the SMR and UMR were around 90% (Phase 1), 82.6% and 81.3% (Phase 2), 82.1% (Phase 3) of the  $Red_{COD}$ , while the CMR was 83.1% (Phase 1), 80.2% (Phase 2), 67.1% (Phase 3) of the  $Red_{COD}$ . Especially during Phase 3, there was a large difference in the  $Red_{COD}$  between the SMR and CMR. The methane production rates of the three reactors increased according to the shortened HRT. In contrast, the  $Me_{COD}$  decreased according to the shortening of the HRT. The methane production rate and the  $Me_{COD}$  of the SMR were clearly larger than those of the UMR under the same HRT conditions, and were moreover, comparable to those of the CMR. This indicates that the siphon installation made a contribution to improving methane yield. In the literature, methane yields from food waste were defined as ml per gram of VS added, and the values previously reported were within 350–450 ml/g-VS added under a long HRT more than 20 days [20,22]. The methane yields of SMR were on average 374 Nml/g-VS added (Phase 1) and 337 N ml/g-VS added (Phase 2), which were almost in the range reported by other researchers. This demonstrates the validity of the performance of the SMR, and verifies that the SMR was able to produce a sufficient amount of methane from the food waste.

**Table 1.** Summary of the digestion performance of the three reactors with the different HRT conditions investigated.

Reactor	SMR				UMR		CMR		
	30	15	10	7.5	30	15	30	15	10
COD in the effluent sludge (g/L)	7.1 ± 1.1	23.0 ± 7.4	18.1 ± 7.7	26.0 ± 4.2	10.9 ± 2.6	22.3 ± 7.9	20.1 ± 1.6	18.3 ± 1.6	33.5 ± 5.2
COD reduction rate ( $Red_{COD}$ ) (%)	93.2 ± 1.4	82.6 ± 5.4	82.1 ± 6.2	73.9 ± 6.0	89.8 ± 2.4	81.3 ± 8.3	83.1 ± 2.7	80.2 ± 4.0	67.1 ± 3.4
Methane production rate (L/L/day)	1.1 ± 0.1	2.1 ± 0.2	2.5 ± 0.3	3.7 ± 0.5	0.9 ± 0.2	1.6 ± 0.3	1.1 ± 0.1	1.8 ± 0.2	2.7 ± 0.3
Methane conversion rate ( $Me_{COD}$ ) (%)	92.1 ± 7.5	83.4 ± 2.1	71.0 ± 10.2	67.9 ± 7.7	75.1 ± 10.9	63.3 ± 14.3	83.6 ± 9.2	81.2 ± 11.5	71.5 ± 3.2
Total COD recovery efficiency ( $Rec_{COD}$ ) (%)	99.0 ± 8.4	102.0 ± 11.2	88.9 ± 9.9	89.2 ± 8.2	85.6 ± 11.9	84.0 ± 17.0	102.0 ± 10	100.3 ± 11.9	102.8 ± 8.0

From what was discussed above, the UMR was characterized by a higher  $Red_{COD}$  and the lower methane production rate than those of the CMR. However, generally  $Red_{COD}$  and methane production are similarly inclined because methane production is mainly responsible for COD reduction in usual anaerobic digestion. In addition, the UMR was marked by the  $Rec_{COD}$ , which was significantly lower than 100%. This suggests the possibility that a part of the organic matter added had been retained in the reactor for a longer time than the HRT. This may explain the higher  $Red_{COD}$  and the lower methane production rate of the UMR. On the contrary, the CMR had almost 100%  $Rec_{COD}$  throughout all the Phases, indicating that the added COD and recovered COD as the effluent and methane were

sufficiently. In the case of SMR, almost 100% of the  $\text{Rec}_{\text{COD}}$  was obtained during Phases 1 and 2, while about 89% of the  $\text{Rec}_{\text{COD}}$  was obtained during Phases 3 and 4. This is probably because there was an accumulation of organic matter as COD inside the reactor during Phase 3 and 4 operations. One of the possible reasons for this COD accumulation has already been described above: the significant scum formation in the SMR may well have been responsible. However, the SMR was found to be able to have a COD recovery as high as the CMR at longer HRTs. In this respect, the SMR has an advantage over the UMR.

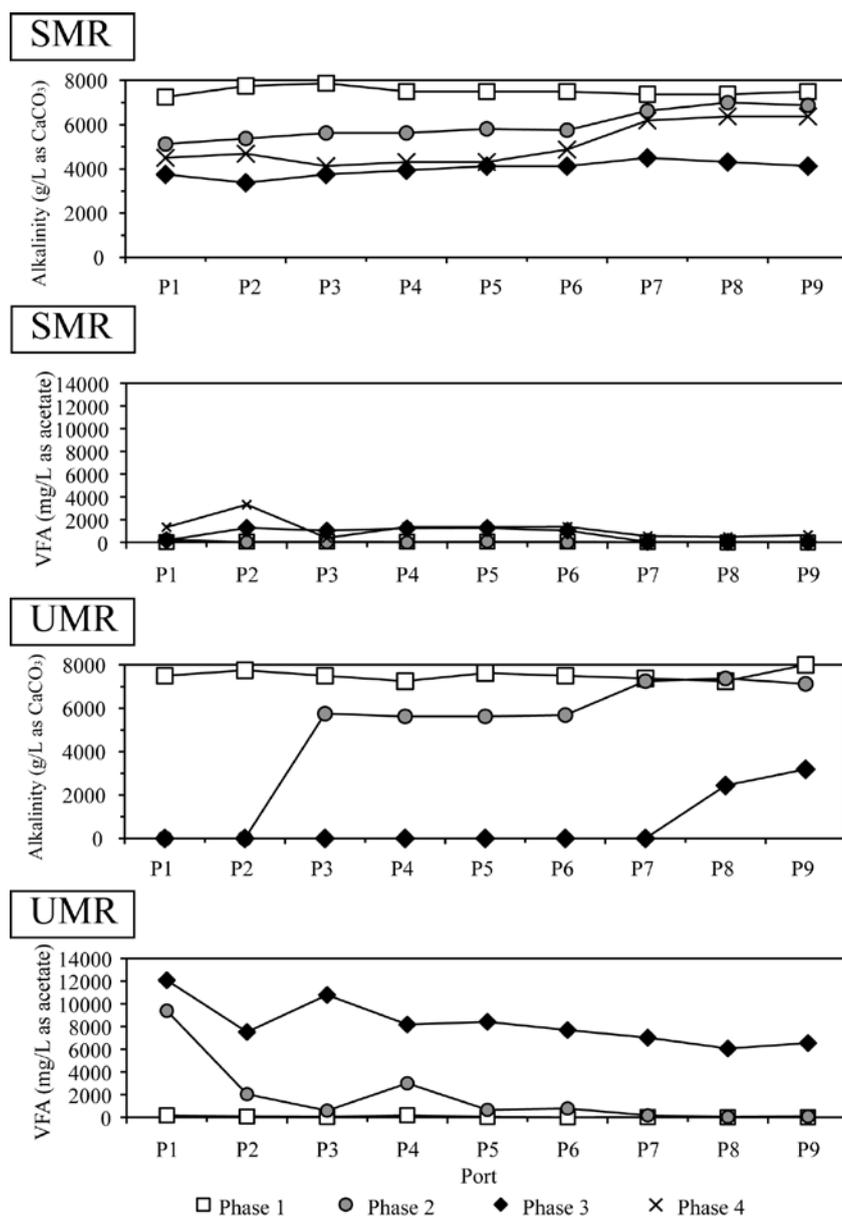
### 2.3. VFA, Alkalinity and Solid Distribution inside The Reactors

The variation patterns of VFA and alkalinity in the SMR and UMR are summarized in Figure 2. The data in the figure were the average values of two or three samples taken during each Phase, respectively. The characters (P1–P9) in the graphs represent sampling ports where the sludge was taken. As clearly shown, there was a great difference in the variation patterns between the SMR and UMR. In the SMR, although the data are not shown in the figure, each of the pH values at the nine ports was the same under the same HRT, and the pH levels slightly dropped from 7.8 to 7.2 on average as the HRT was shortened from 30 days to 10 days, but the pH increased a little again to 7.5 on average when HRT was 7.5 days. The VFA variation pattern in the SMR showed a very similar trend to the pH. The VFA was maintained at low levels in the entire reactor throughout the experiment. Although a little increase in VFA to 3330 mg/L as acetate was observed at P2 during Phase 4, the concentrations were, in most cases, below 1300 mg/L as acetate in the whole SMR. Unlike the VFA, the alkalinity levels of SMR changed depending on HRT. However, under the same HRT, there was no significant difference in the values among P1–P9.

The average alkalinity level decreased according to the shortening of HRT from 30 to 10 days, while the level increased again at HRT 7.5 days, which is a similar trend to that of the pH. Alkalinity is affected by various factors such as  $\text{NH}_4^+$ , dissolved  $\text{CO}_2$  and VFA. The  $\text{NH}_4^+$  and VFA concentration in the reactors were different in the different phases while there was no significant difference in the  $\text{CO}_2$  partial pressure between the four phases. Especially, the  $\text{NH}_4^+$ -N level in the effluent sludge was low (around 1600 mg/L) only during Phase 3, whereas that was within the range from 2600 to 3400 mg/L during the other phases.  $\text{NH}_4^+$  production mostly depend on the anaerobic degradation of organic matter, and therefore, the lower N content in the food wastes used during Phase 3 likely led to a decrease in both the alkalinity and pH level. The VFA/ALK ranges of the SMR were as follows: <0.009 (HRT 30 days); <0.015 (HRT 15 days); 0.011–0.38 (HRT 10 days); 0.075–0.71 (HRT 7.5 days). The VFA/ALK had a tendency to decrease toward the outlet. The VFA/ALK value exceeded 0.4 only at P2 during Phase 4. The SMR was found to maintain stable operation throughout almost the entire period.

On the other hand, the only HRT which resulted in stable operation in the UMR was 30 days in terms of VFA/ALK. Figure 2 shows a significant increase in VFA at Phases 2 and 3 although the VFA levels in the UMR during Phase 1 were as low as those in the SMR. Notably, during Phase 3, the VFA concentrations were above 6000 mg/L as acetate throughout the entire reactor. In addition, the VFA concentration tended to reduce toward the outlet port (from P1 to P9).

Figure 2. Variation patterns of VFA and alkalinity concentrations in the SMR and UMR.

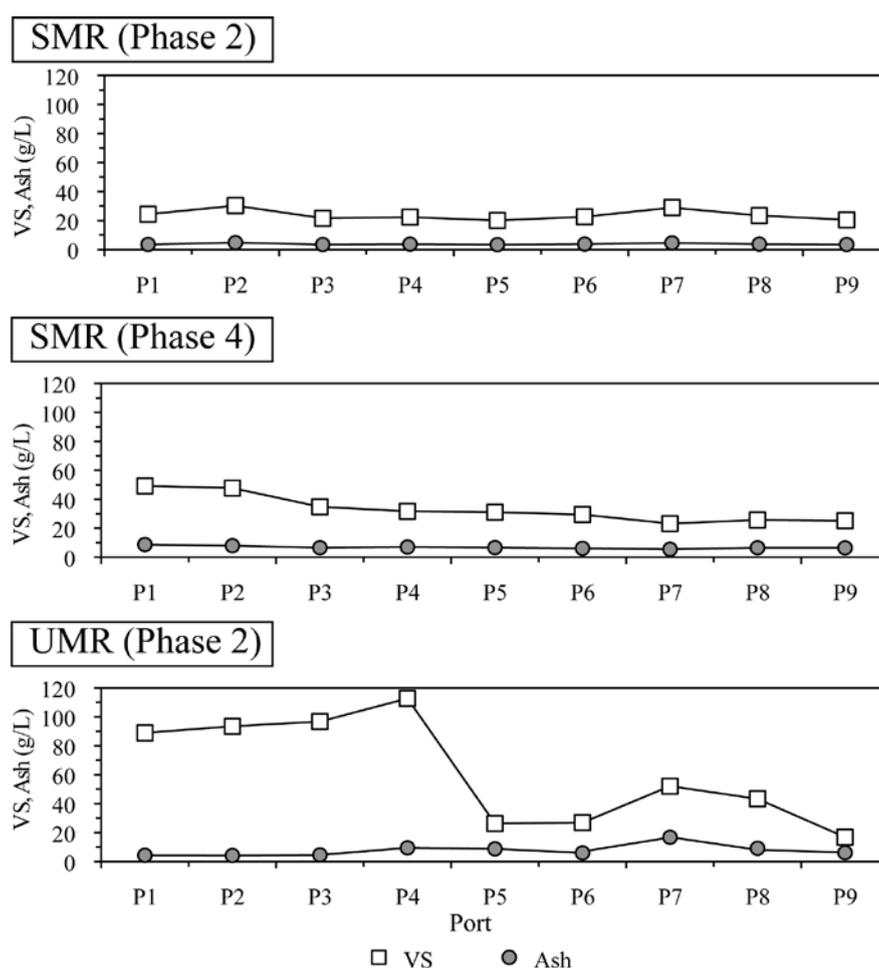


The alkalinity levels in the UMR decreased more sharply than in the SMR as the HRT was shortened. The alkalinity maintained its level in the whole reactor during Phase 1. The VFA/ALK values exceeded 0.4 at three ports during Phase 2, and at all the ports during Phase 3. These results indicate that the UMR was already showing signs that the condition of the reactor would worsen during Phase 2 even though successful operation was maintained in the UMR and methane production was active.

The variation patterns of VS and ash obtained during Phases 2 and 4 are summarized in Figure 3. The results of the Phase 2 shows that the VS concentrations varied from 20.0 to 30.3 g/L in the SMR, and from 16.9 to 113 g/L in the UMR, depending on the place in the reactor. The ash concentrations varied from 3.5 to 4.8 g/L in the SMR, and from 4.2 to 46 g/L in the UMR. What this means is that there was a greater variation of VS and ash in the UMR. In the UMR, the VS concentrations were extremely high at P1, P2, P3 and P4, which are located in the front part of the reactor, while the ash

concentrations were not much different from those at the other ports. This variation pattern implies that the organic materials added were not degraded well and not adequately dispersed. In the rear part of the UMR, the VS and ash levels at P7 and P8 were higher than at the other ports (P5, P6 and P9). Both P7 and P8 were located in the lower part, whereas P5, P6 and P9 were in the upper part. Solid-liquid separation is likely to occur more readily in the rear part because most solid materials have already been degraded in the front part. Therefore, the VS and ash content seemed to accumulate as deposits in the lower part of the C3, where the P7 and P8 ports were located. In the SMR, however, the VS and ash in the SMR were uniformly distributed, suggesting that siphon mixing improved the dispersion of the organic and inorganic solids in the reactor.

**Figure 3.** Variation patterns of VS and ash concentrations in the SMR and UMR.



A comparison between the variation profiles of SMR during Phases 2 and 4 indicates no significant increase in VS in the reactor after long-term operation. It should be noted that the variation patterns found for the SMR with organic solids differed significantly from those of plug-flow reactors previously reported. Langenhoff [23] reported that COD concentrations inside the reactor almost doubled in about 100 days in an anaerobic reactor treating colloidal wastewater. Boopathy [24] showed a vertical VSS distribution in an anaerobic baffled reactor, and a significant accumulation of the VSS in the lower part. The SMR was found to have different distribution characteristics of organic materials in the reactor. From their fluid analysis of the SMR, Qi *et al.* [14] predicted that, the front part would

function much like a CMR and the rear part would function more like an unmixed plug flow reactor. However, our findings demonstrate that the deposition of organic matter was significantly reduced even in the rear part, and as such did not resemble the unmixed reactor. It should be noted that this feature could be well utilized to reduce deposit accumulation and stratification.

#### 2.4. Microbial Community Structure Responsible for Methane Generation

To understand the compositions of methanogenic archaea present in each reactor under stable operation, genomic DNA samples were obtained from the effluent sludge during Phase 2, which was the last period during which all the reactors were able to maintain successful operation. Three 16S rRNA gene clone libraries of the domain *Archaea* were constructed. Table 2 summarizes the operational taxonomic units (OTUs) obtained, and their closest strains in terms of the similarity of partial 16S rRNA gene sequences (around 500 bp in length). The DNA sequences of approximately 30 clones were analyzed for each library. The two libraries of the SMR and UMR were characterized by a predominance of the *Methanosaeta*-related clones as acetoclastic methanogen. By contrast, in the case of CMR, *Methanosarcina*-related clones were predominant in the form of acetoclastic methanogen. The genus *Methanospirillum*, *Methanobacterium* and *Methanoculleus* are known as hydrogenotrophic methanogen. Table 2 indicates that the genus *Methanospirillum* was predominant in the all reactors in the form of hydrogenotrophic methanogen, although the genus *Methanobacterium* was predominant in the UMR. These results suggest that the methanogenic community structure in the SMR is somewhat similar to that in the UMR. It should be noted that a large difference in the archaeal community composition among the three reactors was observed, although the same feedstock was served.

The two genera, *Methanosaeta* and *Methanosarcina*, are the only contributors for acetate to methane conversion. As such, a competition for acetate between the two genera has been widely discussed in pure culture [25], and *Methanosarcina* have an advantage at higher acetate level because *Methanosaeta* has a lower minimum threshold for acetate utilization (0.42–0.6 mg/L) compared to *Methanosarcina* (12–72 mg/L). In this study, the lower acetate levels were observed in the effluent sludge in the SMR and UMR (<50 mg/L) than in the CMR (100–380 mg/L) during the Phases 1 and 2. Therefore, the acetate level is likely one of the most possible reason for the difference in the methanogenic community. The other possible reason is inhibition of the growth of *Methanosaeta* by  $\text{NH}_4^+$  and VFA. Microbial community in full-scale anaerobic digesters has been widely investigated by many researchers. In most cases, the *Methanosaeta*-absence community was formed when the methanogens were inhibited by environmental conditions such as high  $\text{NH}_4^+$  and VFA concentrations [26,27]. In some of the literature, it has been reported that the *Methanosaetaceae* were detected only in reactors with a  $\text{NH}_4^+$ -N concentration below 2 g/L and a VFA concentration below 1.5 g/L as acetate [27,28]. In the case of this study, the  $\text{NH}_4^+$ -N exceeded 3 g/L and the VFA concentration was below 0.2 g/L as acetate during Phase 2 in all the reactors. As such, the reactors were operated under similar conditions in terms of the  $\text{NH}_4^+$  and VFA concentrations. Another possible factor governing the methanogenic community is the mixing condition [29,30]. There was a tendency that the abundance of the *Methanosaeta* showed a marked increase when mixing speed was low (50–60 rpm) or minimal rather than high [29,30]. In this study, the CMR was stirred at 300 rpm and equipped with the impellor twice the diameter of the other researches [29,30]. The strong mixing in the

CMR may have negatively affected the growth of the *Methanosaeta*. Although no sufficient explanation has yet to be offered for the mechanism of the inhibition of *Methanosaeta* under high mixing intensity, what has been discussed above points out the possibility that the difference in mixing intensity between the SMR and CMR made a difference in the methanogenic community.

**Table 2.** Summary of the OTUs obtained from the sludge samples of each reactor investigated.

OTU	Closest strain (Accession number)	Identity	Clones obtained
SMROTU1	<i>Methanospirillum hungatei</i> strain JF1 (NR_042789)	97%	18
SMROTU2	<i>Methanosaeta concilii</i> strain Opfikon (NR_028242)	99%	11
SMROTU3	<i>Methanosarcina thermophila</i> (NR_044725)	98%	1
UMROTU1	<i>Methanosaeta concilii</i> strain Opfikon (NR_028242)	99%	20
UMROTU2	<i>Methanobacterium beijingense</i> (NR_028202)	99%	4
UMROTU3	<i>Methanospirillum hungatei</i> strain JF1 (NR_042789)	97%	3
UMROTU4	<i>Methanosarcina mazei</i> strain DSM 2053	96%	1
UMROTU5	<i>Methanosarcina thermophila</i> (NR_044725)	98%	1
UMROTU6	<i>Methanosaeta concilii</i> strain Opfikon (NR_028242)	90%	1
CMROTU1	<i>Methanosarcina mazei</i> strain DSM 2053 (NR_041956)	98%	18
CMROTU2	<i>Methanospirillum hungatei</i> strain JF1 (NR_042789)	97%	8
CMROTU3	<i>Methanospirillum hungatei</i> strain JF1 (NR_042789)	92%	1
CMROTU4	<i>Methanospirillum hungatei</i> strain JF1 (NR_042789)	92%	1
CMROTU5	<i>Methanoculleus bourgensis</i> strain MS2 (NR_042786)	99%	1

The different influences of a *Methanosaeta*-predominant community and a *Methanosaeta*-absence community on operation have been discussed elsewhere. Karakashev *et al.* suggested that in some of the biogas reactors, the *Methanosarcina*, which is able to utilize acetate, hydrogen, methanol and methylamines, contributed not to the direct conversion of acetate into methane, but rather tended to be contribute to the conversion of hydrogen into methane via the pathway of syntrophic acetate-oxidizing (SAO) to hydrogen [26]. Actually, there have been many reports that the SAO and methane generation via hydrogen was the predominant pathway despite of the presence of the *Methanosarcina* [26,27]. So, there is a tendency that acetoclastic methane generation is more active in the *Methanosaeta*-predominant community, while hydrogenotrophic methane generation is more active in the *Methanosaeta*-absence community. Schnurer and Nordberg reported that the shift from the acetoclastic pathway based on the *Methanosaeta*-predominant community to the SAO pathway led to a decline in the methane yield [31]. By contrast, Hoffmann *et al.* argued that the *Methanosarcina*-predominant community made it possible for the reactor to recover more rapidly from the shock load [30], indicating that the *Methanosarcina*-predominant community was responsible for stable operation, particularly in the start-up. As such, it is still unclear which community is better. Further research is needed on this issue.

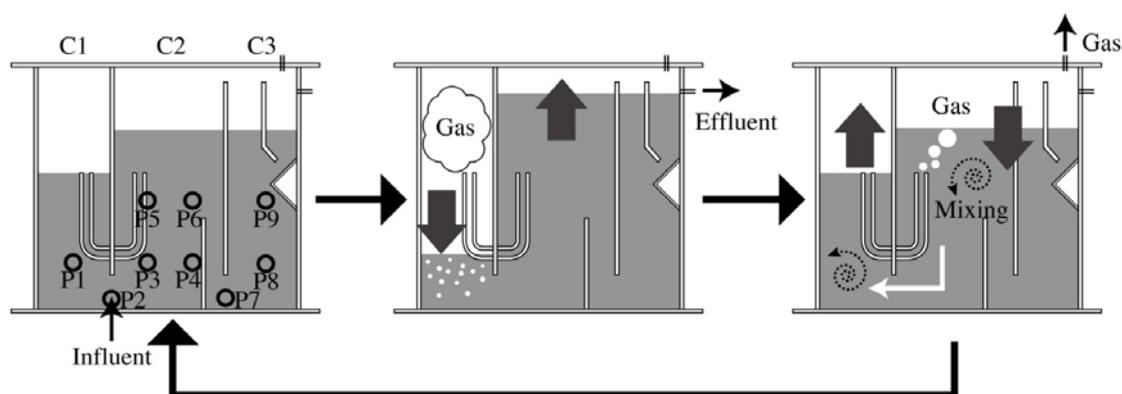
### 3. Experimental Section

#### 3.1. Experimental Apparatus

Three reactors were semi-continuously operated in parallel. The first reactor is the SMR, which is equipped with a siphon tube in the reactor. The second reactor is the unmixed reactor (UMR), which

has almost the same configuration as the SMR, but without a U-tube. Finally, the third reactor is the CMR with a mixer powered by an electric motor. The working volumes of the SMR and UMR were 10 L, and that of the CMR was 5 L. To understand the sludge characteristics at different positions, the SMR and UMR were equipped with nine sampling ports on the side of the reactors, as shown in Figure 4. The CMR was a cylinder with 19 cm in diameter and 30 cm in height, and the mixing was performed by a mixer (Oriental Motor, Speed control motor PSH425, Tokyo, Japan) with a 12 cm diameter axis flow impellor. The stirring speed was 300 rpm. The temperature of each reactor was maintained at 35–36 °C via a water jacket placed around the reactor. A feed tank with a volume of 100 L, which was maintained at 4 °C in the same way as described above, was used for feedstock storage. The feed tank was mechanically stirred by impellers. The mixing speed was 300 rpm.

**Figure 4.** Experimental apparatus and the cycle of the siphon mixing.



In the SMR, mixing was carried out as illustrated in Figure 4. The reactor has three compartments: C1, C2 and C3, as shown in Figure 4. C1 and C2 are linked by two U-tubes in parallel with an inner diameter of 2 cm. The headspace of C1 is closed in the SMR while it is not in the UMR. The biogas pushes the liquid level of C1 down as the gas accumulates in the closed headspace. At the same time, the liquid level in C2 and C3 gradually increases, and the overflow is pushed out of the reactor. When the liquid level reaches the bottom of the U-tubes, the biogas accumulated in C1 is instantaneously transported into C2, causing the liquid in C2 and C3 to be rapidly drawn toward C1. It is this sudden change in the liquid level that stirs the liquid in the reactor.

### 3.2. Feedstock and Seed Sludge

Kitchen waste collected from a cafeteria once in one or two weeks is the feedstock used in anaerobic digestion. After being diluted by a factor of 2.4 with tap water, the kitchen waste was disintegrated by a cutter pump (Toshiba, Tokyo, Japan) and transferred to the feed tank. To supply trace minerals for methanogenic archaea, 100 mg/L  $\text{FeCl}_2$ , 10 mg/L  $\text{CoCl}_2$  and 10 mg/L  $\text{NiCl}_2$  were added to the feed tank. Feeding to the reactors was carried out via time-controlled roller pumps (FURUE Science, RP-LVS, Tokyo, Japan), with a portion of the digested sludge overflowing from the reactors. The average characteristics of the feedstocks were as follows: total solid (TS) was  $100.3 \pm 10.4$  g/L; VS was  $95.1 \pm 10.0$  g/L; COD is  $104.8 \pm 18.6$  g/L. The seed sludge was anaerobic digested sludge obtained from a full-scale anaerobic digester at Sen-en wastewater treatment plant, in Miyagi, Japan.

### 3.3. Chemical Analysis

The gas composition (CH<sub>4</sub>, N<sub>2</sub> and CO<sub>2</sub>) was measured on a daily basis using a gas chromatograph (Shimadzu GC-8A, Kyoto, Japan) equipped with a thermal conductivity detector (TCD) and a stainless steel column packed with Shincarbon ST (Shimadzu GLC, Kyoto, Japan). The temperatures of the column and the detector were maintained at 100 °C and 120 °C, respectively. Biogas production was monitored almost everyday using a wet gas meter (Shinagawa W-NK-0.5, Tokyo, Japan). The COD was measured using the COD Digest Vials (Hach, Loveland, CO, USA) in accordance with the instructions in the manual. TS, VS and alkalinity were determined according to the U.S. EPA Standard Method. Lipid content was measured via a Bligh-Deyers method. The pH was determined using a pH meter (TOA-DKK) equipped with a GST-5721C probe as soon as possible after sampling. The volatile fatty acid (VFA) concentration was determined using a gas chromatograph (Shimadzu, GC14B, Kyoto, Japan) equipped with a flame ionization detector (FID) and a StabilwaxR-DA capillary column (Resteck, Bellefonte, PA, USA). Nitrogen in the form of ammonium ion (NH<sub>4</sub><sup>+</sup>-N) was analyzed using the automatic analyzer Traacs 2000 (Bran + Luebbe K.K., Norderstedt, Germany). Samples for VFA and NH<sub>4</sub><sup>+</sup>-N analysis were prepared by centrifuging sludge samples at 13,000 rpm for 5 min and filtering them through a 0.45 µm pore-size filter. The COD reduction rate (Red<sub>COD</sub>) was defined by following equation:

$$\text{Red}_{\text{COD}}(\%) = 100 \frac{\text{COD}_{\text{inf}} - \text{COD}_{\text{eff}}}{\text{COD}_{\text{inf}}} \quad (1)$$

where COD<sub>inf</sub> is COD in the influent feedstock (g/L); COD<sub>eff</sub> is COD in the effluent sludge (g/L). The methane conversion rate (Me<sub>COD</sub>) was calculated by following equation:

$$\text{Me}_{\text{COD}}(\%) = 100 \frac{R_{\text{CH}_4-\text{COD}}}{Q_{\text{inf}} \text{COD}_{\text{inf}}} \quad (2)$$

where R<sub>CH<sub>4</sub>-COD</sub> is the methane production rate (g/day as COD); Q is the influent flow rate (L/day). The total COD recovery efficiency (Rec<sub>COD</sub>) was defined as follows:

$$\text{Rec}_{\text{COD}}(\%) = 100 \frac{R_{\text{CH}_4-\text{COD}} + Q_{\text{eff}} \text{COD}_{\text{eff}}}{Q_{\text{inf}} \text{COD}_{\text{inf}}} \quad (3)$$

where Q<sub>eff</sub> is the effluent flow rate (L/day).

### 3.4. Cloning Analysis of 16S rRNA Gene

Genomic DNA used for cloning analysis was extracted from the effluent sludge taken from each reactor on the last day of Phase 2. DNA extraction was performed using a PowerFood Microbial DNA Isolation Sample Kit (MO-BIO, Carlsbad, CA, USA). The extraction protocol was according to the manual of the kit. Amplifications of the DNA by polymerase chain reaction (PCR) were performed with the primers A109f [32] and 1510r [33] specific for the 16S rRNA gene of *Archaea*. DNA amplification was carried out under the following conditions: initial denaturing for 5 min; 30 cycles of the PCR, which consisted of denaturing for 30 s at 94 °C, annealing for 40 s at 53 °C, extension for 2 min at 72 °C; final extension for 5 min at 72 °C. The PCR product was cloned with a TOPO TA Cloning kit (Life Technologies, Carlsbad, CA, USA). The cloned DNA fragments obtained from

randomly selected recombinants served as templates for the sequencing analysis. The sequencing was performed at Dragon Genomics Center (Takara Bio, Yokkaichi, Japan). The OTU was defined as a cluster of aligned sequences with 97%–100% similarity.

#### 4. Conclusions

Considering the results of this study, the following conclusions can be drawn: (1) Siphon mixing enhances digestion performance in terms of acceptable OLR and methane yield compared to the unmixed reactor; (2) Siphon mixing promotes the dispersion of solid materials and reduces deposits in the reactor; (3) The methanogenic community of SMR was dominated by the genus *Methanosaeta* as acetoclastic methanogen, which was absent in the community formed in the CMR.

#### Conflict of Interest

The authors declare no conflict of interest.

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