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Abstract

This study investigated the impact of mixing on key factors including foaming, substrate stratification, methane production and microbial community in three full scale anaerobic digesters. Digester foaming was observed at one plant that co-digested sewage sludge and food waste, and was operated without mixing. The lack of mixing led to uneven distribution of total chemical oxygen demand (tCOD) and volatile solid (VS) as well as methane production within the digester. 16S rRNA gene-based community analysis clearly differentiated the microbial community from the top and bottom. By contrast, foaming and substrate stratification were not observed at the other two plants with internal circulation mixing. The abundance of methanogens (*Methanomicrobia*) at the top was about four times higher than at the bottom, correlating to much higher methane production from the top verified by *ex-situ* biomethane assay, causing foaming. This result is consistent with foaming potential assessment of digestate samples from the digester.

Keywords: Anaerobic co-digestion; Foaming; Digester mixing; Biomolecular techniques; Microbial community.

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1. Introduction

Utilising the spare digestion capacity at wastewater treatment plants (WWTPs) for co-digestion of sewage sludge with organic wastes can simultaneously allow for energy self-sufficiency and sustainable waste management (Nghiem et al., 2017; Xie et al., 2018). This approach, known as anaerobic co-digestion (AcoD), has been actively considered and pursued by many water utilities around the world. There have been a number of full-scale AcoD implementations with demonstrated technical and economic success (Nghiem et al., 2017; Shen et al., 2015). However, recent research results and full scale experience also highlight a number of key bottlenecks hindering the implementation co-digestion to existing WWTP facilities (Nghiem et al., 2017). Lack of a design guideline can potentially lead to operational disruptions caused by organic overloading, substrate inhibition, and digester foaming that are associated with AcoD (Mata-Alvarez et al., 2014; Mehariya et al., 2018; Meyer & Edwards, 2014; Xie et al., 2018). Previous studies have focused on controlling organic loading in AcoD through substrate selection and adjusting mixing ratio between sewage sludge and organic wastes (Ma et al., 2017; Nghiem et al., 2014; Wickham et al., 2016; Zhang et al., 2019) and on elucidating substrate inhibition mechanism on digester performance via microbial community analysis (Li et al., 2015; Nguyen et al., 2018; Razaviarani & Buchanan, 2014). Relatively less engineering controls are known to alleviate foaming, which is a common but has rarely been investigated problem in AcoD (Hagos et al., 2017; Kougias et al., 2015; Nghiem et al., 2017).

Digester foaming causes both operational and economic issues for AcoD process. Foaming can decrease the effective digester volume by up to 50%, reduce biogas production by up to 40%, cause digester overflow, damage equipment and affect performance of biological nutrient removal as well as dewaterability of digested sludge (Ganidi et al., 2009; Moeller et al., 2018; Tyagi et al., 2018). While it is not always possible to accurately calculate the

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financial cost of foaming, digester foaming has been accounted for 150,000 USD/year in economic loss in a WWTP in Sweden (Kougias et al., 2015). Digester foaming is complex and it is not always easy to identify the direct cause as well as mitigation strategy. In most cases, anaerobic digester foaming is caused by a combination of several supplementary factors that is initiated by one primary cause (Ganidi et al., 2011; He et al., 2017; Kougias et al., 2013).

Digester mixing is an important operating condition with potential ramification on foaming (Subramanian et al., 2015; Tyagi et al., 2018). Digester mixing enables homogeneity between substrates and microorganisms for digestion (Kaparaju et al., 2008; Lindmark et al., 2014; Stroot et al., 2001). Without mixing (w/o mixing), a localized build-up of nutrients (Moeller et al., 2012; Subramanian & Pagilla, 2015) and sludge (Stroot et al., 2001) was observed in the digester. Even mixing also provides sufficient contact amongst four microorganism groups to ensure smooth transfer of substrate from one group to another within the digester. For instance, the metabolic activities of two major microorganism groups i.e. acetate-foaming bacteria and methane-forming archaeal require close spatial contact (Chojnacka et al., 2015). Digester mixing releases gaseous products i.e. CH_4 and CO_2 preventing their accumulation in sludge. However, full-scale study on the impact of digester mixing in AcoD on key factors such as foaming, methane production, stratification and microbial community is not yet available. The conditions such as methane production, stratification and microbial community induced by mixing are probably supplementary factors for foaming formation.

This study aims to investigate systematically the key conditions in a full-scale AcoD that currently operates w/o mixing. Sludge samples from the digester top and bottom locations were analysed to indicate the digester stratification of substrate and foaming potential. Methane productions in the digester was *ex-situ* investigated through a biomethane potential assays using sludge samples form digester top and bottom as inocula. 16S- rRNA gene-based

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community was employed to reveal the microbial distribution within digester. Additional two full-scale anaerobic digesters, which were able to mix sludge, were also subjected to the rigorous analysis for references. Results from this study provide a guidance document on AcoD foaming prevention.

2. Materials and Methods

2.1. Selection of full-scale anaerobic digesters

Three full-scale WWTPs (denoted as plant A, B and C) in New South Wales (Australia) were selected to allow for a strategic analysis of the impact of mixing on digester foaming potential, digester stratification, biogas production and microbial community. It is noted that there was no mixing in the digester at plant A. By contrast, internal circulation was used to provide mixing in plant B and C at the rate of 6 and 5 volume turn-overs per day, respectively. The digester at plant A co-digested of a mixture of food waste and sewage sludge, thus, its organic loading rate was higher than that of both plant B and C (Table 1). Severe digester foaming has been regularly reported at the primary digester at plant A. Plant B has also experienced some occasional and mild digester foaming in the primary digester. Foaming incident has not been reported at plant C. Arrangement of the anaerobic digestion process at these WWTPs is in series and schematically described in Fig. 1. Other characteristics and operational conditions of the digesters were presented in Table 1. Sludge samples were collected from the primary digester at two different locations, namely digester top and bottom. All digesters in this study had cylindrical shape with conical bottom and a floating roof. Sampling point at the bottom was approximately one meter above the conical body of the digester. Sampling point at the top was approximately one meter below top of the digester at plant B and C through established sampling valves. At plant A, sludge samples from the top were collected through the gap between the digester main body and the

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floating roof. Two sampling events (referred to as sampling event #1 and sampling event #2) were conducted at each plan within 20 days apart.

[TABLE 1]

[FIGURE 1]

2.2. Foaming potential evaluation

The aeration method from Subramanian et al., (2015) was used to assess the foaming potential of digested sludge samples. The foaming potential apparatus consisted of the following: air pump, diffusing air-stone, flow meter and a 2 L graduated cylinder. Fresh sludge was collected from the top and bottom of the primary digesters and subjected immediately to foaming potential assessment. The sludge (200 mL) was transferred into a graduated cylinder. The diffusing air-stone was placed at the bottom of the cylinder. Then, the initial height of sludge was recorded in volume (mL) or in height (cm) in 2 L graduated cylinder. An air pump was set to provide airflow of 1.5 L/min into sludge. Under aeration, foam tended to build up in the cylinder as air bubbles were created. The sludge was aerated for 30 min or until the highest level of foam was observed. Then, the air pump was stopped for one minute and the level of foam was recorded. This test was used to characterize two types of foams: unstable and stable. Unstable foam collapsed once the air supply was stopped within one minutes, while stable foam persisted. Unstable and stable foam ratios were calculated according to Equations 1 and 2 respectively. The resultant foaming potential ratio was assessed follow foaming potential thresholds (Table 2) (Subramanian et al., 2015).

$$\text{Unstable foam ratio} = \frac{Maximum\ foam\ height\ (mL)}{Initial\ height\ of\ sludge\ (mL)} \quad (1)$$

$$\text{Stable foam ratio} = \frac{Settled\ foam\ height\ (mL)}{Initial\ height\ of\ sludge\ (mL)} \quad (2)$$

[TABLE 2]

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2.3. Biomethane potential assay

The impact of mixing on biogas production in the AD was assessed *ex-situ* using a customized biomethane potential (BMP) system (Wickham et al., 2016). The BMP system included an array of 1 L fermentation glass bottles and a gas collection gallery. The fermentation bottles were submerged in a water bath (Model TWB-20D Thermoline Scientific Pty Ltd) to maintain a constant temperature of 35.0 ± 0.5 °C. Each bottle setup comprised of a rubber stopper, a water-filled S-shaped airlock and a valve. Biogas from the bottle could flow through the airlock into the gas collector via flexible plastic tubing. The gas collector was an inverted plastic measuring cylinder (1,000 mL), which was initially filled with, and partially submerged in, a 1 M NaOH solution.

Digested sludge samples from the top and bottom of each digester were collected into 5 L plastic containers and used as inoculum after 2 h of collection. Prior to all BMP experiments, fermentation bottles were flushed with pure N_2 for 5 min before filling with 400 mL inoculum and feed (3:1 v/v). Then, the bottle was flushed again with N_2 and immediately sealed with the rubber stopper. The bottles were then placed into a water bath pre-heated to 35.0 ± 0.5 °C. Biogas from the fermentation bottles was introduced into the submerged part of the cylinder, thus allowing the NaOH solution to absorb CO_2 and H_2S from the biogas. The remaining CH_4 gas displaced the NaOH solution inside the cylinder and the CH_4 gas volume generated was recorded daily. The experiment was terminated after 20 days when less than 10 mL/day of CH_4 was produced. BMP experiments were conducted in triplicate.

The effect of different inoculum to methane production in BMP was analysed using the modified Gompertz model as shown below.

$$M_P = P_m * exp\left[-exp\left(\frac{R_m}{P_m}(\delta - t\right) * e + 1\right]$$

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Where, Mp was the cumulative methane production (mL), Pm was ultimate methane production (mL), Rm was the methane production rate (mL/day), δ was the lag-phase time (day) and e was the exponential.

2.4. Volatile solids and total chemical oxygen demand

Volatile solids (VS) and total chemical oxygen demand (tCOD) of digested sludge samples from the top and bottom were measured in this study to quantify the stratification of the digesting sludge within the digesters. The resultant data were used to calculate the VS and tCOD ratio between sludge sample form the top and bottom. These ratios can show the stratification within the digesters. VS was measured following the APHA Standard Method 2540. tCOD was measured by using a HACH digestion vials and a HACH DR3900 spectrophotometer following the manufacturer's instruction. All analyses were carried out in triplicate.

2.5. Microbial community analysis

Microbial community of digested sludge samples from the top and bottom of each digester was analyzed in this study. Digested sludges from the top and bottom were collected into 50 mL sample bottle and mixed with 100% ethanol (1:1 v/v) to preserve the cells. Further details about sample preparation are available elsewhere (Luo et al., 2016). Briefly, samples were stored in an ice bag during transport and immediately transferred to - 20 °C freezer upon arrival to laboratory. Genomic DNA was extracted using DNeasy PowerSoil Pro Kit (QIAGEN Pty Ltd, Australia) following the manufacture's instruction. The integrity, purity and concentration of the extracted DNA were determined by a spectrophotometer (Nanodrop ND2300). The mass of DNA in each sample was always more than 10 µg and the concentration was normalized to 50 ng/µL using DNA/RNA free water. Samples were stored at - 20 °C until DNA sequencing.

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The variable regions (V3-V4) on the 16S rRNA gene of extracted DNA were amplified using the universal primers Pro341F (*5'-CCTACGGGNBGCASCAG-3'*) and Pro805R (*5'-GACTACNVGGGTATCTAATCC-3'*) (Takahashi et al., 2014). The amplified fragments were sequenced on Illumina MiSeq sequencing platform at the Australian Genome Research Facility, Australia. Raw paired-end (2×300 bp) 16S rRNA gene sequence data were analyzed according to the Quantitative Insights into Microbial Ecology (QIIME2) pipeline (Caporaso et al., 2010). In brief, raw sequences were denoised using DADA2 with the following parameters: trim left-f = 17, trim left-r = 20, trunc-len-f = 280, trucn-len-r = 220, and all other parameters at their default setting. The sequences were clustered into representative OTUs based on a 97% nucleotide identity cutoff. The 16S rRNA gene sequencing generated 120,000 to 450,000 sequences per sample after preprocessing. Taxonomical assignment was performed against MiDAS database version 2.1 (McIlroy et al., 2017). The 16S rRNA gene sequences were deposited in GenBank with the accession numbers PRJNA507317. Non-metric multidimensional scaling analysis, canonical correspondence analysis and compositional similarity index were performed in PASS software with Bray-Curtis index. Statistical analysis was performed in Microsoft Excel using Student's unpaired *t*-Test, with a two-tailed distribution and in PASS using a permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001).

3. Results and Discussion

3.1. Foaming potential

Mixing plays an important role affecting the difference in foaming potential between the top and bottom of the digester (Fig. 2). There was no mixing in the digester at plant A, thus, there was a marked difference in the foaming potential between the top (i.e. severe foaming) and bottom (non-foaming), respectively. These results indicate the accumulation of foaming associated substances such as hydrophobic surface-active agents at the top of digester w/o

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mixing. Although the surface-active agents were not analyzed in this study, proteins, volatile fatty acids, detergents, lipids and biosurfactants are amongst active agent compounds in anaerobic digestion (Ganidi et al., 2009). The substances could come from the feed or the metabolism of microorganisms in anaerobic digestion. Accumulation of surface-active agent at the air and liquid interface could be eliminated by maintaining a well-mixed digester. High concentration of hydrophobic substances also contributes to a stabilized layer of foam as indicated by a similarity between stable and unstable foaming (Fig. 2). The observed high COD and VS concentration (Table 3) at the top of the digester and high foaming potential at plant A could have a cause and effect relationship. On the other hand, foaming potentials were homogenous at the digesters at plant B and C with digester mixing. Sludge samples from the top and bottom of digester at plant B were characterized severe-foaming and mild-foaming potential according to the unstable and stable foaming thresholds (Table 2). The digester at plant C has a non-foaming potential according to both stable and unstable foaming thresholds (Table 2). The observation of foaming potential at the digester at plant B suggests that there is a critical concentration of surface-active agents to induce or stabilize foaming during the anaerobic digestion process. However, analytical method of surface-active agents is not readily available, thus, quantitative assessment of foaming via the aeration method in this study is necessary.

[FIGURE 2]

Results obtained from foaming assessment are consistent with the actual condition of these digesters during this study and their historical data described in section 2.1. During this study, no foaming incidents were observed at plant C. At plant B, there was an ongoing foaming episode at the aerobic biological treatment process. The carryover waste activated sludge from the foam aeration tanks after thickening (ca. 23% of daily feed volume) to the digester could contribute to the observation of mild-foaming from both foaming test and the plant event with

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mixing. At plant A, although there were different foaming potentials at the top and bottom of the digester, severe-foaming potential at the top in the foaming test was consistent with high risk of foaming occurrence in the digester at plant A. The results from this study suggest that foaming potential assessment is a valuable tool for routine monitoring of the risk of digester foaming at full-scale anaerobic digesters.

Solid-liquid phase separation and accumulation of foaming associated substances on the top of digester due to mixing are the main reasons form foaming incidents (Ganidi et al., 2009; Moeller et al., 2018; Stroot et al., 2001). In laboratory scale experiment, Stroot et al. (2001) observed a foam layers on the liquid surface, which was about 50% of digester volume, during minimal mixing condition (i.e. twice per day by manual hand shaking for two minutes). This phenomenon could become more severe if mixing was not provided in the digester such as at full-scale plant A. The effect of mixing on foaming in the AD was also more intense in the presence of cofactors such as high organic loading rate, high solid content of the feed and temperature fluctuation. This is evident in the digester at plant A. Historically, the digester at plant A was operated with mono-digestion (i.e. digestion of sewage sludge). No foaming episodes were observed during mono-digestion. Recently, food waste was introduced as co-substrate (2% daily feed volume) into the digester which increased the organic loading rate from 3.6 to 4.1 kg VS/m^3/d during the course of this study. Although the organic loading could be a contributing factor to foaming at plant A, the lack of mixing was likely the main cause of consistently high foaming potential. Overall, the results indicate the need for adequate mixing to mitigate digester foaming at plant A.

3.2. Substrate distribution within the digester

Digester mixing has significant impact on substrate distribution in the three digesters in this study. W/o mixing, the substrate (in terms of VS and tCOD) distribution within the digester varied significantly between the top and bottom. At plant A, in the absence of mixing, the VS

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and tCOD ratios between sludge from the top and bottom were 2.0 ± 1.3 and 2.6 ± 0.6, respectively, indicating the accumulation of VS and tCOD at the top of the digester (Table 3). In addition, the VS/TS ratio of sludge sample from the top of digester w/o mixing suggests high level of VS component. On the other hand, the VS and tCOD ratios were approximately 1 at plant B and C since there was digester mixing at both of these plants. The VS and tCOD profile in the digester at plant A was inverse of what could be expected in a gravity tank. This observation could be the result of flotation effect causing by gas production during the anaerobic digestion.

[TABLE 3]

Stratification has been identified as a major issue during AD operation (Ghanimeh et al., 2012; Karim et al., 2005). The uneven distribution of substrates can result in over- and under-loading zones within the digester. The former leads to issues with organic loading while the latter leads to under-utilization of the digester capacity. Kaparaju et al. (2008) observed stratification during the non-mixing period with the lighter fraction on the surface (by floatation) and heavier solids at the bottom (by gravity). Stroot et al. (2001) observed that total solid level of sludge sample at the top was four-times higher than the bottom of digester with inadequate mixing.

In addition to uneven substrate distribution, stratification could result in the accumulation of surface active agents and hydrophobic compounds such as protein, volatile fatty acids, detergents and lipids on the surface of an inadequate mixing digester (Ganidi et al., 2009). By experimental addition of lipid compound (e.g. sodium oleate), Kougias et al. (2013) observed the accumulation of lipid on the top of digester caused foaming formation. Similarly, Boe et al. (2012) suggested that high content of lipids and protein promote foaming in the anaerobic digester. Therefore, the stratification of substrate with possibly high level of surface active agents could induce severe-foaming potential of sludge at the top of digester such as at plant

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A. Furthermore, the availability of substrate due to stratification in the digester could affect the microbial activity, composition, and consequently overall digester performance.

3.3. Methane production within the digester

BMP results indicated a marked difference in methane production from sludge samples taken from the top and bottom of the digester w/o mixing (Fig. 3). The BMP bottles inoculated with sludge samples from the top of the digester produced 1.4 times higher methane than that from the bottom at plant A. Statistical analysis revealed that the methane production was consistently significant with $P < 0.05$ by Student's *t*-test in both sampling event 1 and 2. On the other hand, the average methane production observed in BMP test with sludge samples from both the top and bottom of the digesters at plant B and C were similar (Fig. 3). The results implied that methane production could be at higher rate at the top of the digester w/o mixing. Consistently, the specific methanogenic activity (i.e. production volume and rate) calculated using the modified Gompertz model was higher in the BMP bottles inoculated with sludge samples from the top than that of the bottom of digester w/o mixing. The effect of mixing on biogas production has been reported previously mainly based on the comparison between presence/absence of mixing in the digesters (Ghanimeh et al., 2012; Kaparaju et al., 2008). Although, the results plausibly indicated better performance in mixed digester, no or minimal effect have also been reported. The degree of mixing effect depends on OLR and solid content.

[FIGURE 3]

3.4. Microbial community structure

Sludge samples from the top and bottom of the digester w/o mixing show distinctively different microbial community structure. Non-metric multidimensional scaling (NMDS) analysis shows that the community from the digester top clustered in one group that was separated from the cluster of the community from the digester bottom at plant A (Fig. 4). On

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the other hand, microbial communities of sludge samples from the top and bottom of the digesters at plant B and C were clustered closely (Fig. 4), suggesting high level of community structure similarity. Indeed, the Bray-Curtis similarity index indicated 0.67 ± 0.05, 0.79 ± 0.13 and 0.77 ± 0.1 between microbial communities of sludge samples from the top and bottom of the digesters at plant A, B and C, respectively. A permutational multivariate analysis of variance (PERMANOVA) test revealed that the microbial community structure of the top and bottom of the digester at plant A was significant difference (Bonferroni-corrected $P = 0.02$). The digesters at plant B and C, however, the Bonferroni-corrected P was 0.25 and 0.11, respectively, suggesting no significant difference in microbial community structure between the top and bottom sludge samples in the digester with mixing.

[FIGURE 4]

Analysis of the phylogenetic structure of microbial community conclusively indicated the difference between sludge samples from the top and bottom of the digester at plant A. Significant differences in the abundance of some major bacterial and archaeal classes in the top and bottom the digester at plant A were observed, while the digesters with regular mixing (plant B and C) showed no difference in microbial community at the top and bottom (Fig. 5). Notably, the methanogens (class of *Methanomicrobia*) were four-time more abundant at the top than at the bottom of the digester at plant A (Fig. 5). High abundance of methanogens at the top could be partially attributable to the better methane production observed in the BMP test (Fig. 3). Further analysis of *Methanomicrobia* in sludge samples from the digester top of plant A indicated that the major methanogens belong to the order of *Methanosarcinales*. This order is dominant in the digester with high acetate levels (McMahon et al., 2001; Nguyen et al., 2018). Indeed, the phenotype of *Methanosarcinales* species is aceticlastic methanogens. The increase in methanogens in the top of the un-mixed digester was associated with a significant increase in the abundance of *Actinobacteria* and *Deltaproteobacteria*. The bacteria

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from both classes are likely in syntrophy with the methanogens by providing them with substrates like acetate. Indeed, some *Actinobacteria* (like *Micrococcus*) can be involved in acidogenesis (the conversion of soluble organic molecules into acetate, hydrogen and carbon dioxide), while *Deltaproteobacteria* such as *Pelobacter* sp. have been reported to oxidize ethanol into acetate and hydrogen in syntrophic cooperation with methanogens (Schmidt et al., 2014).

Proliferation of filamentous bacteria in the class of *Actinobacteria* has been identified as a possible cause of foaming in activated sludge due to their filament structure and hydrophobic cell walls (Guo et al., 2015; Petrovski et al., 2011). However, the relative abundance of *Actinomycetales* order of *Actinobacteria* class was below 0.2% in sludge samples from all three digesters in this study. Thus, filamentous microorganisms may not be a cause of foaming in the digesters. The results implied that providing even distribution of microorganisms through mixing of digester should be considered to insure proper operation of the AD process without compromising the overall microbial community structure.

[FIGURE 5]

3.5. Relationship between foaming and other operating parameters

Results reported from the CCA analysis highlight the relationship between foaming and substrate distribution, methane production as well as microbial community profile (Fig 6). In the absence of mixing in the digester at plant A, the CCA indicated a positive correlation between VS concentration, COD concentration, CH_4, microbial communities and foaming potential at the top of the digester (Fig. 6). On the other hand, microbial communities at the bottom of digester negatively correlated with these parameters. Methane production, microbial communities at the top of the digester at plant A and foaming potential showed a closely positive correlation with each other. The correlation also coincided with the observed methane production and foaming potential (Section 3.1&3.3). It is noted that biogas

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contributes up to 95% of foam layer in the digester. Under the high gas production rate, foam has higher tendency to form in the AD (Etoc et al., 2006; Vardar-Sukan, 1998). With favorable conditions in none mixing digester such as in the digester at plant A (i.e. high VS and COD at the top), biogas bubbles may accumulate at the liquid surface faster than they decay, leading to foam formation. The CCA plot also implied that there was no correlation amongst these relevant parameters in the digester at both plant B and C that have mixing of sludge (Fig. 6). Therefore, maintaining homogenous conditions in the digester through regular mixing is suggested to avoid foaming formation and operational issues.

[FIGURE 6]

4. Conclusion

The lack of mixing can lead to uneven distribution of tCOD, VS and methane production within the digester. The top of digester has high concentration of tCOD and VS that is probably the result of floatation effect. Higher methane production at the top than bottom was observed in an *ex-situ* biomethane assay. Consistently, different microbial community was revealed with significantly high abundance of methanogens at the digester top. The conditions induced by w/o mixing are probably contributing factors to the observed severe-foaming potential at the top of digester. The results initiated the provision of mixing in operation of the digester.

Appendix A. Supplementary data

E-supplementary data of this work can be found in online version of the paper.

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List of Figure:

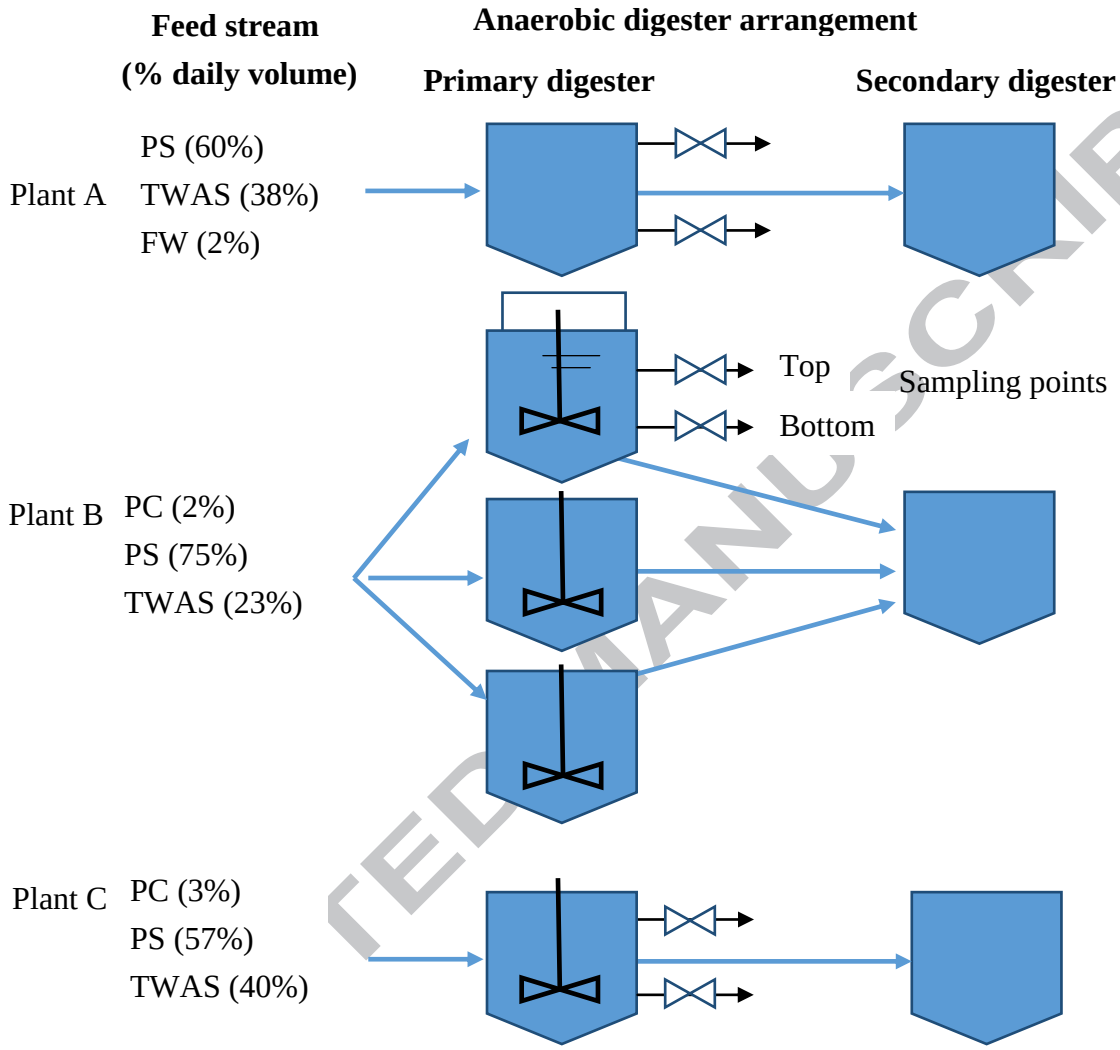

Fig. 1 Feed stream and anaerobic digester arrangement at plant A, B, and C in this study (PS = primary sludge; TWAS = thickened waste activated sludge; FW = Food waste; PC = Primary scum).

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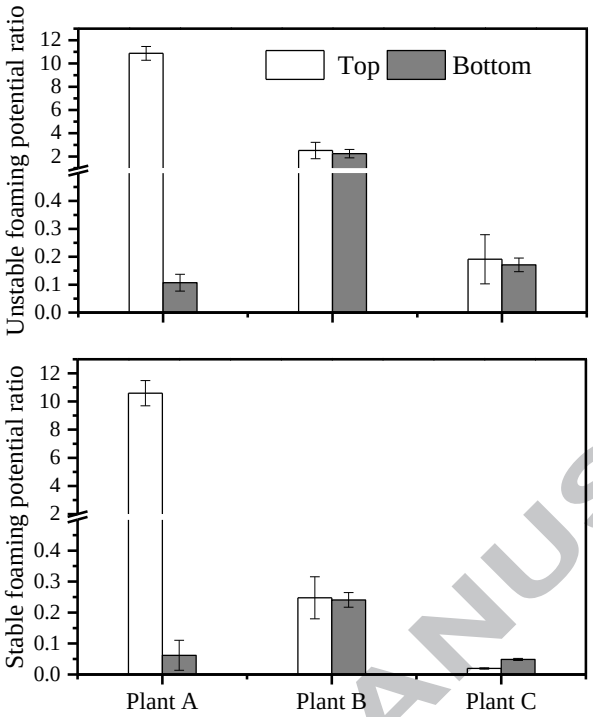

Fig. 2 Unstable and stable foaming potential ratio at three plants. Value and error bars are mean and standard deviation (*n* = *4*).

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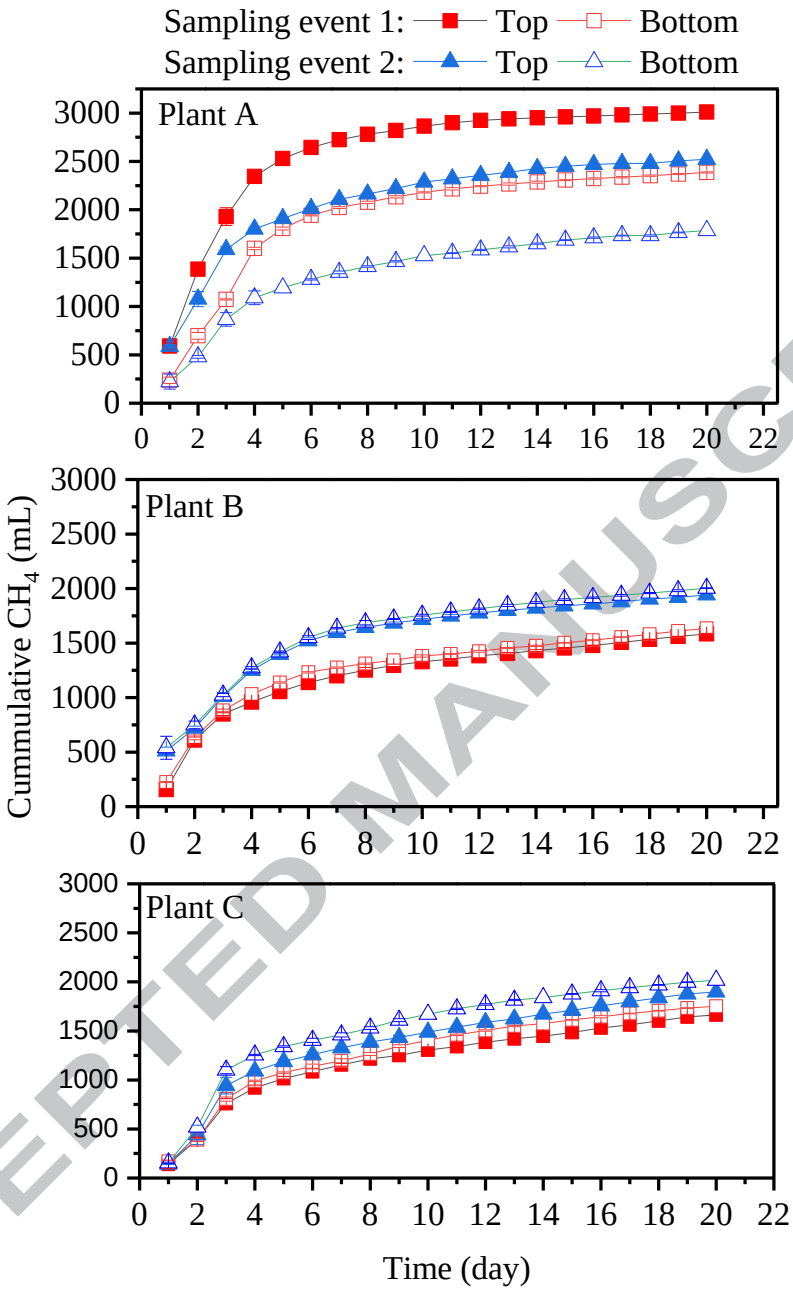

Fig. 3 Cumulative methane production over time in BMP tests with digested sludge samples taken from the top and bottom of the digesters. Value and error bars are mean and standard deviation (*n* = *3*).

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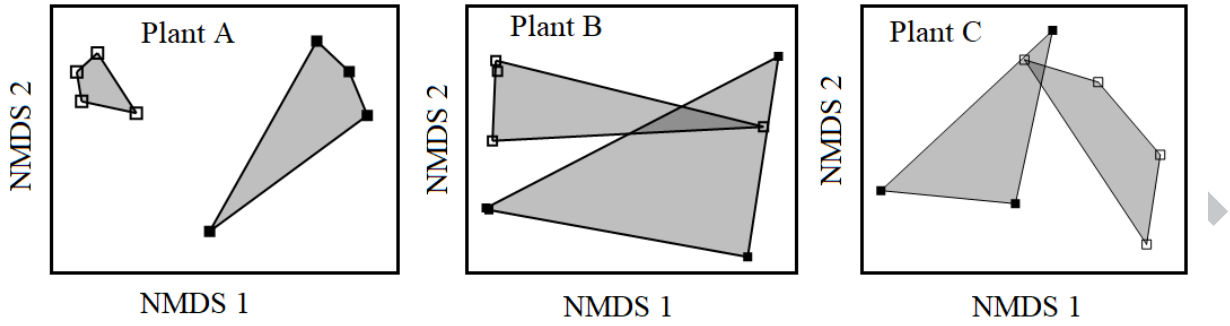

Fig. 4: Non-metric multidimensional scaling (NMDS) ordination of the bacterial and archaeal community structure from sludge samples taken from the top (fill square) and bottom (open square) of the digesters at plant A, B and C.

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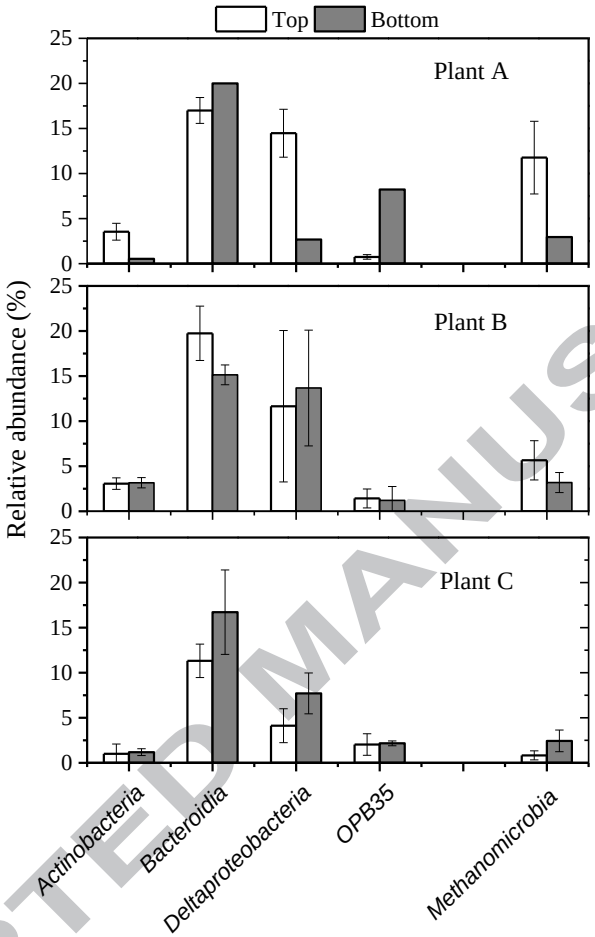

Fig. 5 Major bacterial and archaeal class relative abundance that showed statistical significance between communities from sludge samples from the top and bottom of the digester at plant A (with $P < 0.05$ by Student's t-test). Value and error bar are mean and standard deviation ($n=4$).

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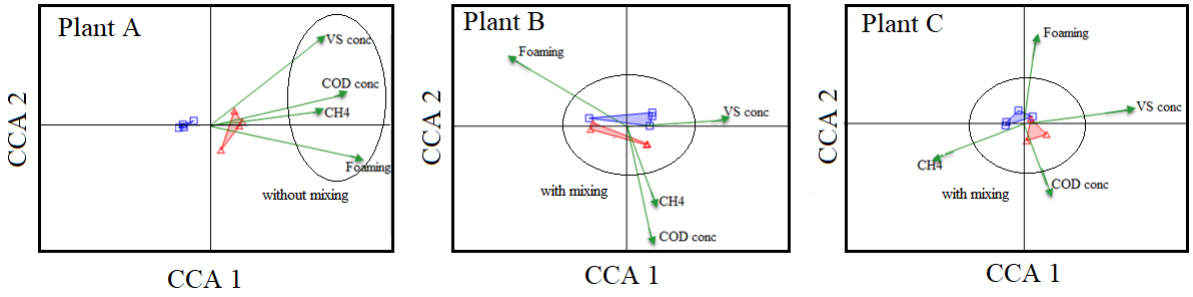

Fig. 6 Canonical correspondence analysis (CCA) ordination of the foaming potential, substrate distribution, methane production and microbial community. Open squares and triangles represent the communities from the top and bottom of the digester respectively. The vectors represent the influence of mixing on microbial community clustering.

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List of Table:

Table 1: Full-scale anaerobic digester characteristics

Parameter	**Plant A**	**Plant B**	**Plant C**
No. of digesters	2	4	2
Digester arrangement in series	One primary One secondary	Three primary One secondary	One primary One secondary
SRT (day) in the primary digester	10	7	11
Temp (°C)	37	35.6	33
Digester capacity (ML per digester)	4.25	2.99	3.6
OLR (kg VS/m^3 day)	4.1	1.66	3.57
Total COD in feed (kg/m^3)	131 ± 12	38 ± 1.7	42 ± 2.7
Mixing	No[#]	Yes	Yes

[#] The only form of mixing in plant A occurs when sludge and food waste are fed to the digester.

Table 2: Foaming potential assessment thresholds

Scale	**Non-foaming**	**Mild foaming**	**Severe foaming**
Unstable foam	0 – 1	1 – 2	> 2
Stable foam	0 – 0.2	0.2 – 0.5	> 0.5

Table 3: VS and tCOD concentration of sludge from the top and bottom of the digester. Data are mean ± standard deviation (*n = 6*).

Site	Digester location	VS (g/L)	tCOD (g/L)	VS/TS ratio	VS_{top}/VS_{bottom}	$tCOD_{top}/tCOD_{bottom}$
Plant A	Top	46 ± 19	121 ± 3	0.92 ± 0.11	2.0 ± 1.3	2.6 ± 0.6
	Bottom	23 ± 2	49 ± 2	0.76 ± 0.07		
Plant B	Top	22 ± 3	44 ± 6	0.72 ± 0.02	0.93 ± 0.0	1.0 ± 0.0
	Bottom	25 ± 2	43 ± 6	0.75 ± 0.09		
Plant C	Top	25 ± 3	40 ± 2	0.75 ± 0.06	0.98 ± 0.0	1.1 ± 0.0
	Bottom	24 ± 2	38 ± 1	0.76 ± 0.09		

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Highlight

- W/o mixing, foaming potential was severe at the top of the digester
- Much higher tCOD/VS concentration and methane production at the top w/o mixing
- W/o mixing, NMDS analysis clearly separated microbial communities from top/bottom
- W/o mixing, methanogens were highly abundant at the top of the digester
- Lack of mixing is likely to be the primary cause of digester foaming

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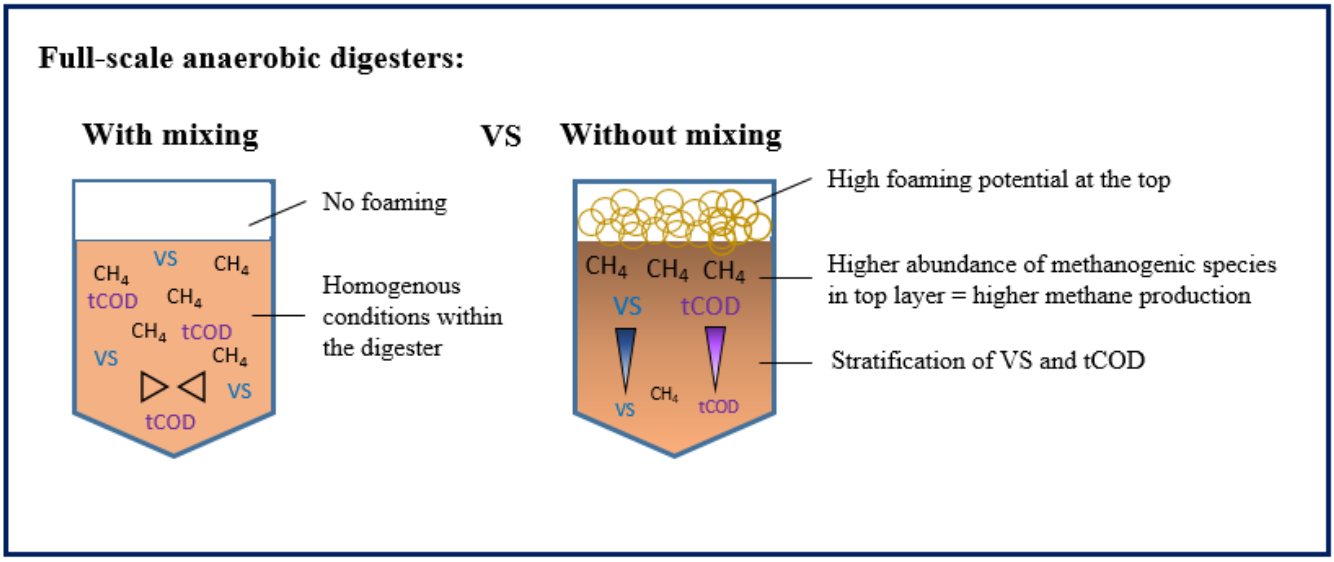
Graphical abstract



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