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Title: Liquid digestate recycled utilization in anaerobic digestion of pig manure: Effect on methane production, system stability and heavy metal mobilization

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Abstract: To improve bioenergy, methane, production in the anaerobic digestion plant application, the effects of recycled liquid digestate on anaerobic digestion of pig manure were investigated. Two continuous stirred tank reactors were operated for 230 days with varying organic loading rates (OLRs, from 1.5 to 6 g VS L-1 d-1); one reactor was implemented with liquid digestate recirculation and the other was set as the control without recirculation. It was demonstrated that the recirculation operation improved methane production and system fermentation stability, particularly for OLRs below 5 g VS L-1 d-1. The inhibition of methane production was found under an OLR of 6 VS L-1 d-1, which was caused by significantly increased viscosity from 30 to 1000 mPa·s and decreased mass transfer characteristics. The previously reported negative effects of accumulated ammonia and VFA on anaerobic digestion under digestate recirculation were not found in the present investigation of pig manure treatment. However, the heavy metals Pb, Mn, Cu and Zn accumulated in both liquid and solid fractions of the generated digestate in the digestate recycled reactor. The stable carbon isotope analysis of □13CCO2 and □13CCH4 produced the biogas may indicate different methanogenic pathways between the anaerobic reactors with and without digestate recirculation.

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Cover Letter

Beijing, 13.03.2017

Dear Editor,

We hereby submit the enclosed manuscript entitled “Liquid digestate recycled utilization in anaerobic digestion of pig manure: Effect on methane production, system stability and heavy metal mobilization”, to be considered for publication in *Energy*.

To improve bioenergy, methane (biogas), production in the anaerobic digestion plant application, the effects of recycled liquid digestate on anaerobic digestion of pig manure were investigated. The operatized operation mode (digestate recirculation) of pig manure anaerobic digestion to improve the methane production and system stabilization were confirmed. However, the heavy metal accumulation and methanogenic pathways were shifted after the operation. The finding can further inspire the application. The paper help to develop the renewable energy production, optimize the energy processes, and mitigate the environmental pollutants. Thus, the paper perfectly fit the aims and scope of this journal.

We thank you for considering our manuscript and we hope it will be of your interest. All authors have seen this manuscript and approved to submit to your journal. This paper describes an original work and it is not being submitted to any other journal. The manuscript has been checked by English native speakers with expertise in the field.

Looking forward to hearing from you, I remain respectfully yours.

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Highlights

- Digestate recycling on AD can promote renewable energy production under low OLR
- Digestate recycling can improve the AD system stability under low OLR
- Increased viscosity decreased mass transfer and lowered renewable energy production
- Stable isotopic analysis of biogas indicated different methanogenic pathways
- Heavy metal accumulation under digestate recycling may influence land application

Liquid digestate recycled utilization in anaerobic digestion of pig manure: Effect on methane production, system stability and heavy metal mobilization

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Abstract

To improve bioenergy, methane, production in the anaerobic digestion plant application, the effects of recycled liquid digestate on anaerobic digestion of pig manure were investigated. Two continuous stirred tank reactors were operated for 230 days with varying organic loading rates (OLRs, from 1.5 to 6 g VS L^{-1} d^{-1}); one reactor was implemented with liquid digestate recirculation and the other was set as the control without recirculation. It was demonstrated that the recirculation operation improved methane production and system fermentation stability, particularly for OLRs below 5 g VS L^{-1} d^{-1}. The inhibition of methane production was found under an OLR of 6 VS L^{-1} d^{-1}, which was caused by significantly increased viscosity from 30 to 1000 mPa·s and decreased mass transfer characteristics. The previously reported negative effects of accumulated ammonia and VFA on anaerobic digestion under digestate recirculation were not found in the present investigation of pig manure treatment. However, the heavy metals Pb, Mn, Cu and Zn accumulated in both liquid and solid fractions of the generated digestate in the digestate

recycled reactor. The stable carbon isotope analysis of $\delta^{13}C_{CO_2}$ and $\delta^{13}C_{CH_4}$ produced the biogas may indicate different methanogenic pathways between the anaerobic reactors with and without digestate recirculation.

Keywords: Biogas; Process stability; Recirculation; Swine manure; Stable isotope

1. Introduction

Anaerobic digestion (AD) has been recognized as a cost-effective biological technology for bioenergy production using various bio-waste, such as livestock manure [1, 2]. The traditional crude oil consumption has reached 388 million t in 2009 in China, and over 50% of that was imported, thus, Chinese government is heavily promoting the renewable energy production plant to offset the energy needed [3-4]. The renewable energy production has accounted around 10% of the China's primary energy consumption, and the contribution is tremendously increasing in these years [3]. China, as one of the leading pork production countries in the world, has built more than 90,000 AD plants with a total treatment capacity of 14 million m^3 [4]. Thereafter, large amounts of anaerobic digestate have been generated as by-products from AD plants along with the energy production. The digestate is rich in nutrients and traditionally used as bio-fertilizer for agriculture [2]. However, the quantity of digestate production often exceeds the consumption ability of surrounding farmland, and digestate transport from the point of surplus to distant farmlands is not economically feasible. More important, the anaerobic digestate always still contain considerable amounts (25-30%) of methane potential under short hydraulic retention time in AD plant [5]. Thus, in order to fulfil the throughout energy recovery and methane production, the proper disposal method of AD plant digestate is urgently required.

The recycled utilization of digestate has preliminarily been demonstrated as an efficient method for recovering energy and reducing digestate discharge from some AD

plants [5-8]. The recycling operation could introduce the residual methane potential and methanogenic bacteria back to the AD plant, which can theoretically significantly improve the methane production of the applied AD plant. However, the concerns surrounding recycling of digestate in AD plants are the accumulation of key chemical inhibitors to methane production in AD plant, such as ammonia and volatile fatty acids (VFAs) [9, 10]. It is commonly accepted that excessive ammonium accumulation can increase the proportion of free ammonia, which is toxic for methanogenesis and results in lower methane production. However, this process might also be highly dependent on feeding materials. For example, Estevez et al., 2014 [7] observed a 16% increase in methane yield with liquid digestate recycling of cow manure with Salix, whereas Wu et al., 2016 [8] reported a decrease of 43% in methane production under liquid digestate recycling of chicken manure due to ammonia accumulation. In addition, viscosity may also increase with digestate recirculation and decrease methane production due to imperfect substrate movement in AD plants. Until now, the implications of digestate recirculation have been studied in AD of dairy manure [6], chicken manure [8] and co-digestion of cow manure with salix [7] or grass silage [5]. To our knowledge, the renewable energy, methane, production performance of pig manure anaerobic digestion under digestate recycling operation has not been studied.

Besides the energy production efficiency, the deep understanding of methane generation mechanisms change under the digestate recycled utilization would be important to support the recycling application in AD plant. Molecular analysis methods, such as next generation high-throughput sequencing, can accurately identify the dominant methanogenic *Archaea* [11], but are insufficient for quantifying specific metabolic activity [12]. Carbon isotope analysis has become an important tool for studying specific metabolic activity from hydrogenotrophic and acetotrophic methanogens in AD plants [13, 14]. It is well accepted that methane (CH_4) is primarily produced from acetate by acetotrophic methanogens (Eq. 1) and from CO_2 and H_2 by hydrogenotrophic methanogens (Eq. 2) in AD

reactors. Additionally, formate may also play an important role as a substrate (Eq. 3) for formatotrophic methanogens [15]. Notably, formate (Eq. 3) usually acts as a precursor for hydrogenotrophic methanogens (Eq. 2) due to CO_2 production.

$$CH_3COOH \rightarrow CH_4 + CO_2 \quad (1)$$

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad (2)$$

$$4HCOOH \rightarrow 3CO_2 + CH_4 + 2H_2O \quad (3)$$

General criteria has demonstrated that methanogenesis by CO_2-reduction (Eq. 2) will lead to strongly depleted $\delta^{13}C_{CH4}$ values in comparison to methanogenesis by acetate cleavage (Eq. 1). The fractionation factor of $\delta^{13}C_{CO2}$ and $\delta^{13}C_{CH4}$ ($aC = (\delta^{13}C_{CO2} + 10^3) / (\delta^{13}C_{CH4} + 10^3)$) is another possible indicator to approximately identify dominant methanogenic pathways. Published literature suggests that lower aC values indicate that acetotrophic methanogens would be the more dominant pathway of methane production in AD reactors [16]. The inorganic and organic compounds, as well as bacterial biomass, will refill AD plants after digestate recycling operations, which might change the methanogenic pathways [17]. Nevertheless, the impacts of digestate recirculation on methane production mechanisms in AD reactors have never been investigated.

Another knowledge gap hypothesis is the dynamics of heavy metals in digestate from AD plants might also influence the operation of digestate recirculation; heavy metal concentration will regulate the digestate application by agriculture. Thus, to achieve the full success application of this optimized digestate recycled AD plant, the accumulation and mobilization of heavy metals in digestate effluent under liquid digestate recirculation need to be monitored.

The aims of the study presented here were to assess the liquid digestate recirculation operation on the energy production performance and mechanisms of pig manure anaerobic digestion. For this purpose, laboratory-scale CSTRs for treating pig manure at variable OLRs (increased from 1.5 to 6 g VS L^{-1} d^{-1}) were implemented under

mesophilic conditions. The influences of AD reactor digestate recirculation on methane production, and the characteristics of the digested substrate, were investigated to evaluate methane production and system stability. Additionally, to assess the risk potential for agricultural utilization of the final digestate effluent, the dynamics of heavy metals accumulation and mobilization were determined in both the liquid and solid fractions. Moreover, to understand the impact of recirculation on predominantly methanogenic pathways, the composition of $\delta^{13}C_{CO_2}$ and $\delta^{13}C_{CH_4}$ associated with the molecular analysis method were monitored.

2. Material and methods

2.1 Materials and inoculums

The pig manure used in this study was collected from a larger-scale pig farm located in Beijing, China. The raw pig manure was naturally dried, followed by pulverization treatment. Then, the homogenized samples were frozen at -20 °C to prevent biological decomposition. The frozen substrates were thawed in a refrigerator at 4 °C for 1 day prior to introduction to the reactor. The sludge inoculum was collected from a biogas plant (located in Shun Yi district, Beijing, China) with mesophilic pig manure AD by CSTRs. The characteristics of the pig manure and inoculum sludge are summarized in Table 1.

2.2 Experiment set-up and operation

Two laboratory-scale CSTRs were implemented in two identical glass cylinders with a total volume of 15 L and effective volume of 10 L. The feeding and discharge ports were set at the top and bottom of the reactors, respectively. The gas outlet was at the top and connected with a plastic gasbag. Both CSTRs were intermittently stirred at 120 r/min for 1 h on and 1 h off. The whole experiment lasted 230 days, and the experiment was maintained at 37 ± 1 °C in a temperature-controlled chamber. Once a day, approximately 660 mL of digestate was drawn out through the outlet port at the bottom of the CSTRs, and the same amount of raw material was fed in via the feeding port. The hydraulic

retention time (HRT) of both CSTRs was maintained at around 15 days through the entire experiment. To investigate the effect of various OLRs and recirculation on the performance of the CSTR reactors, the experiment was divided into two phases with eight OLR levels (Fig. 1). The initial OLR was 1.5 g VS L^{-1} d^{-1} during the start-up experimental period. Then, the OLR gradually increased until reaching 6 g VS L^{-1} d^{-1}. In phase I, both CSTR reactors (R1 and R2) were established without recirculation until day 57 under an OLR of 2.5 g VS L^{-1} d^{-1}. In phase II, R2 was set to 60% effluent liquid digestate recirculation from day 58 to the end (day 230) while R1 was un-recirculated as the control.

2.3 Sampling and analysis

The pH was determined using a digital pH meter (FE20, Mettler-Toledo, Switzerland). The methane production volume was measured with a wet-type precision gas meter (LML-1, Changchun, China). Moreover, CH_4 and CO_2 contents were analysed using a biogas analyser (Eheim Visit 03, Messtechnik Eheim, Germany). Total solids (TS), volatile solids (VS) and total ammonia nitrogen (TAN) of all samples were determined using standard methods [18]. Total inorganic carbon (TIC) and volatile fatty acids (VFAs) were analysed by titration with 0.1 NH_2SO_4 to endpoints of pH 5.0 and 4.4, following the procedure of Zhang et al., 2014 [19]. The sludge viscosity was determined using a rotational viscometer (NDJ-1, Shanghai China) at a shear rate of 60 min^{-1} as described by Chang et al., 2007 [20]. Cellulose and hemicellulose were determined using the sequential analysis method developed by Soest et al., 1985 [21]. All the above parameters were collected from each sample every 1-2 days for routine analyses in the present study.

Additionally, the concentrations of heavy metal elements, including lead (Pb), manganese (Mn), copper (Cu) and zinc (Zn), in both liquid and solid fraction of the effluent were determined using inductively coupled plasma mass spectrometry (ICP–MS, Elan 9000, Perkin Elmer, USA) approximately every 2 days from day 100 to 230 in the experiment. The stable isotopic enrichments of CH_4 and CO_2 were carried out as described by Nikolausz et al., 2013 [13]. Briefly, an isotope ratio mass spectrometry system (Finnigan MAT 253,

Thermofinnigan, Bremen, Germany) was coupled to a gas chromatograph (HP 6890 Series, Agilent Technology, USA) via a combustion device for carbon analysis. The isotope analyses were conducted on day 47 and 118 with six replicates of each measurement to represent the performance in phases I and II, respectively.

2.4 Microbial community analysis

In order to investigate the microbial difference between reactors, 16S rDNA characterization was carried out to analyse the microbial communities at the end of the experiment. A sample (20 g) of substrate from each CSTRs (R1 and R2) were used to extract microbial DNA using the FastDNA® SPIN Kit (MP Biomedicals, Santa Ana, CA) according to the manufacturer's instructions. The extracted soil DNA was first detected by agarose gel electrophoresis (1.0% agarose in 0.5× TAE) to examine its integrity and approximate concentration. Then, the quality and quantity of DNA samples were determined using a NanoPhotometer® Spectrophotometer (IMPLEN, Carlsbad, California, USA) and a Qubit® RNA Assay Kit in a Qubit® 2.0 Fluorometer (Life Technologies, Carlsbad, California, USA). The 16S rDNA gene amplification was carried out according to Kozich et al., 2013 [22]. Briefly, the extracted DNA was amplified with a universal primer set (314F/805R) targeting the V3+V4 hypervariable region. The detailed PCR conditions were adopted as described by Kozich et al., 2013 [22]. The amplicons were purified using a Wizard®SV Gel and PCR Clean-up System (Promega, Madison, Wisconsin, USA) after gel extraction. The purified 16S rDNA amplicons were sequenced using the Illumina Miseq platform (Illumina Inc., San Diego, CA) at Anoroad Bio. Tech. Inc. (Beijing, China). After trimming the low quality sequences, residual sequences were aligned using MOTHUR. The aligned sequences were checked for chimera using USEARCH 6.1 in QIIME and classified into Operational taxonomic units (OTUs) within a 0.03 difference (97% similarity) using the de novo OTU picking workflow in QIIME.

2.5 Statistical analysis

Differences between parameters from R1 (control) and R2 (recirculated reactor)

were evaluated using the repeat measured student T test at 95% confidence level ($p < 0.5$) of significant differences. Principal Component Analysis (PCA) was used to identify different treatment performance patterns between R1 and R2 under each experimental phase with different OLRs. PCA was conducted using all measured parameters, which includes pH, methane production, VFA, TIC, TAN, viscosity, TS, VS, hemicellulose and cellulose. For PCA analysis, data was standardized (to a Z score with a mean = 0 and S.D. = 1) to ensure that each variable had the same influence in the analysis. Sigmaplot software (version 12.5, Sigma, Inc.) and XLStat Pro® (XLStat, Paris, France) were used for plotting and data analyses, respectively.

3. Results

3.1 Methane production

The methane production of the two CSTR reactors (R1 and R2) was compared as time progresses in the experiment (Fig. 2). Operation conditions were the same for R1 and R2 during phase I, and methane production performance were also similar between the two reactors ($P > 0.05$). Both daily (Fig. 2a) and volumetric methane production (Fig. 2b) increased along as OLRs increased with value ranges from approximately 2-5 L d^{-1} and 0.5-0.9 m^{-3} m^{-3} d^{-1}, respectively. Subsequently, in phase II, R2 changed to the liquid digestate recirculation mode and R1 was maintained in the un-recirculated mode. Methane production (both daily and volumetric) increased in R2 compared to R1 until the OLRs increased to 5 g VS L^{-1} d^{-1}. The average daily and volumetric methane production reached 11 L d^{-1} and 2.0 m^{-3} m^{-3} d^{-1} for R1 and 13 L d^{-1} and 2.2 m^{-3} m^{-3} d^{-1} for R2, respectively. When the OLR increased to 6 g VS L^{-1} d^{-1}, the methane production fluctuated strongly in R2. Average values decreased to around 11 L d^{-1} and 2 m^{-3} m^{-3} d^{-1} for daily and volumetric methane production, respectively, which were even lower than that of R1. Additionally, regarding methane and CO_2 content, no obvious difference was found between two reactors ($P > 0.05$). The average methane content of R1 and R2 was around 61.2%, and the corresponding average CO_2 content was about 36.9% throughout the entire experiment

(Fig. 2a).

3.2 Anaerobic digestate characteristics

3.2.1 Dynamics of pH, TAN, VFA and TIC

The pH, TAN, VFA and TIC were similar between the two reactors in phase I, with average values of 7.1, 600 mg L^{-1}, 350 mg L^{-1} and 3800 mg L^{-1}, respectively (Fig. 3). However, all these parameters increased tremendously in R2 after liquid digestate recirculation and reached 7.5, 1600 mg L^{-1}, 1200 mg L^{-1} and 1000 mg L^{-1} for pH, TAN, VFA and TIC, respectively. Notably, pH in R2 stabilized at 7.5 after OLR reached 4 g VS L^{-1} d^{-1}. TAN concentration in R2 decreased to around 600 mg L^{-1} when the OLR reached 6 g VS L^{-1} d^{-1}. Nevertheless, these values were significantly higher in R2 as compared to R1, which only showed a slight improvement with increasing OLRs. At the end of this experiment under an OLR of 6 g VS L^{-1} d^{-1}, the pH, TAN, VFA and TIC in R1 achieved 7.2, 600 mg L^{-1}, 600 mg L^{-1} and 5800 mg L^{-1}, respectively.

3.2.2 Dynamics of viscosity, cellulose and hemicellulose, VS and TS

The viscosity, cellulose and hemicellulose, VS and TS were also similar between the two reactors in phase I (Fig. 4). However, it is clear that the R2 viscosity increased exponentially up to around 1000 mPa·s, while it still kept below to 30 mPa·s in R1 as the OLRs increased from 2.5 to 6 g VS L^{-1} d^{-1} in phase II (Fig. 4a). The average cellulose and hemicellulose contents in the R1 and R2 digestate reached up to 4.1% and 9.8%, respectively (Fig. 4b). The average VS (Fig. 4c) and TS (Fig. 4d) contents achieved 6.7 % and 4.2 % for R1, 9.9% and 15% for R2, respectively, at the end of the experiment.

3.3 Performance patterns of the two CSTR reactors

Methane production (MP), as well as the substrate characteristics, including pH, VFA, TIC, TAN, viscosity, TS, VS, hemicellulose and cellulose (H&C) of both CSTR reactors throughout the experiment were analysed using principal component analysis (PCA) to assess the performance patterns (Fig. 5). Independent PCA analyses were completed

separately for experimental phases I (Fig. 5a) and II (Fig. 5b). The first two principal components accounted for a variation of 64.9% and 79.0% for phases I and II, respectively. In phase I, the R1 and R2 patterns generally overlapped and did not show clear group differences under each OLR (Fig. 5a), suggesting the two systems had good parallelism. However, in phase II clear group differences between R1 and R2 were found (Fig. 5b). Both R1 and R2 showed the same tendency and indicated an up-right direction as the OLRs increased. Moreover, the methane production also showed a direct loading effect. However, R1 moved close to and was concentrated in the positive direction of PC2 and R2 moved close to and was concentrated in the positive direction of PC1 along as OLR increased. The values of pH, TAN, TIC, VFA, viscosity, TS and VS showed high positive loadings on the PC1 axis, which indicates a positive correlation with R2 with increasing OLRs (Fig. 5d).

3.4 Heavy metal mobilization

The heavy metals, including Pb, Mn, Cu and Zn, were analysed in both the solid and liquid fractions of the anaerobic digestate effluent for both R1 and R2 in experimental phase II (Fig. 6). All of these heavy metals were generally more than 10 times higher in the solid than liquid fraction for both R1 and R2. Moreover, the contents of Pb, Mn, Cu and Zn in the solid fraction digestate of R2 were significantly higher than R1, and reached approximately 0.7, 90, 120 and 300 mg L^{-1}, respectively. However, the heavy metals in the liquid fraction were only slightly higher (not significant) in R2 as compared with R1. The concentrations of Pb, Mn, Cu and Zn were maintained below approximately 0.08, 4, 8 and 9 mg L^{-1}, respectively, for both R1 and R2.

3.5 Microbial community

The microbial community diversity in R1 and R2 were analysed and compared using 16S rDNA Pyrosequencing. After quality trimming, 15,998 and 16,134 valid Pyrosequencing reads of the 16S rDNA gene were obtained for R1 and R2, respectively (Table S1). Rarefaction curves, based on the OTUs at 3% dissimilarity (Fig. S1), indicated

that the sequences were sufficient to reflect the diversity of the microbial communities. The microbial diversity (Shannon and Simpson index) was similar in R1 and R2 (Table S2). The microbial communities of both reactors showed high diversity at the levels from phylum, class (Fig. S2) to genus (Fig. 7). *Bacteroidetes* and *Firmicutes* were the two dominant phyla; *Bacterioidia* and *Clostridia* were the two dominant classes in both CSTR reactors (Fig. S2).

As shown in Fig. 7, at a genus level, the dominant genus in R1 was *Clostridium* (accounting for 43.3%) which belongs to the phyla *Firmicutes*. Moreover, *SMB53* (16.2%) and *YRC22* (12.2%) were next most dominant genus types in R1. However, the genus *Candidatus Cloacamonas* and *Syntrophus* were the dominant bacterial communities in R2, accounting for 36.2% and 22.4%, respectively. The corresponding values were significantly lower in R1, accounting for 7.1% and 2.1%, respectively.

3.6 $\delta^{13}C_{CH_4}$ and $\delta^{13}C_{CO_2}$ compositions

The isotopic compositions of ^{13}C in CO_2 and CH_4 were analysed and the fractionation factor of aC was calculated for biogas production in phases I and II (Table 2). In phase I, the average values of $\delta^{13}C_{CO_2}$ and $\delta^{13}C_{CH_4}$ for both R1 and R2 were similar and maintained approximately 6‰ and -44‰, respectively. As the OLRs increased from phase I to phase II, the averaged of $\delta^{13}C_{CO_2}$ and $\delta^{13}C_{CH_4}$ in R1 changed to 5.03‰ and -42.20‰, respectively. For R2 in phase II, the averaged of $\delta^{13}C_{CO_2}$ and $\delta^{13}C_{CH_4}$ in R1 changed to 6.40‰ and -48.21‰, respectively. The average fractionation factors of $\delta^{13}C_{CO_2}$ and $\delta^{13}C_{CH_4}$ for R1 were 1.052 and 1.049 in phase I and II, respectively. However, the values for R2 were 1.052 and 1.057 in phase I and II, respectively.

4. Discussion

The main objective of this study was to provide the first comprehensive assessment of the performance anaerobic digestion of pig manure with liquid digested recirculation. In the present study, all parameters for both R1 and R2 were similar in experimental phase I.

The results demonstrated that the two AD reactors performed well as parallel, thus, the two reactors were comparable in phase II when R2 was modified to digestate recirculation mode. An increased pH was observed in both R1 and R2, which was attributed to the increasing OLRs applied to system [23]. The pH value is a pivotal factor that may affect methane production efficiency. However, the pH in both R1 and R2 was in the range of 6.5-7.5 until the end of the experiment, which was still suitable for active methane-forming microorganisms [24].

When the OLR was below 5 g VS L^{-1} d^{-1}, higher methane production was clearly found in R2 as compared with R1 (Fig. 2a). The results indicate that recirculation promoted AD system efficiency under relative low OLRs. It was also demonstrated by the previous study from Estevez et al., 2014 [7], methane yields increased 16% compared to the control in co-digestion of cow manure and Salix under an OLR of 2.6 g VS L^{-1} d^{-1}. The methane was generated from the degradation of biodegradable organic substances in the AD reactor and the methane production can be used to indicate AD system efficiency. The explanation for the increased methane production may be an incomplete degradation of organic substances and biofilm reintroduced to the reactor during recycling [7, 17].

The inhibition of methane production due to ammonia and VFA accumulation was not found, which may due to the pig manure material used for AD in the present study. It has been shown that ammonia accumulation can introduce negative effects on methane production in plant AD [9]. A rise in TAN level with initiation of digestate recirculation has been observed in investigations of mesophilic AD of silages using stirred tank reactors [17], and hyper-thermophilic-mesophilic AD of waste activated sludge in two-stage systems [25]. TAN concentration accumulation is also heavily aggravated with digestate recirculation operation in AD of chicken manure [8]. In the present study, the TAN level in AD effluent increased with the initiation of digestate recirculation. However, the levels were still far below an inhibitory concentration, suggested as above 3000 mg/L, and showed no signs of inhibiting the system throughout the experiment. An inhibition due to accumulated VFA in

an AD reactor with recirculation was found in a previous study [10]. However the accumulated VFA in this study was also lower than the limit concentration, suggested as 2000 mg HAC/L [26], which provided AD stability in the present study.

Additionally, the potential for improved system stability has been found in pig manure AD under recirculation operations. AD system stability can be enhanced by incremental alkalinity (TIC) concentration as well as lower VFA/TIC ratios, which are the most important contributors to buffering capacity in the AD process [24]. In this study, the R2 VFA/TIC ratios maintained 0.11 ± 0.03, which indicates a strong buffering capacity during digestate recirculation. The improvement of system buffering capacity and pig material properties can be explained by the recycling digestate.

Methane production in R2 under digestion recirculation showed strong fluctuations and the average value was lower than R1 under the highest OLR of 6 g VS L^{-1} d^{-1}. Because the TAN and VFA concentrations were clearly below inhibitory concentrations, the hypothesis for methane production inhibition is high viscosity. The TS, VS, cellulose and hemicellulose contents contributed to high viscosity in the AD reactor [27]. The continually increasing content of such factors were especially apparent in R2 under recirculation. The viscosity increase during the AD processes causes a foaming problem by decreasing the mass transfer ability [28]. The digested recirculation might accelerate the accumulation process, because part of the recalcitrant compounds remained in the discharged digestate was recycled and thus accumulated in the recirculating reactors [7]. Therefore, it is crucial to decrease the TS, VS, cellulose and hemicellulose contents and maintain an appropriate viscosity level in the AD reactor for methane production.

The accumulation of heavy metals in agriculture soil due to long-term land application of digestate is a cause for concern because of potential environmental risks. The contents of Pb, Mn, Cu and Zn were 10 times higher in the solid fraction digestate than the liquid fraction digestate in this study, similar to values reported by Zhang et al., 2011 [29]. In addition, the Pb, Mn, Cu and Zn contents in both the solid and liquid fraction

digestate of R2 was higher than R1, showing that heavy metal accumulation was promoted by the digestate recirculation in the AD reactor. The results indicate that the effluent coming from a recirculating system may post a threat to the environment due to the high content of heavy metals. Consequently, to protect the soil environment, it is advisable to test for heavy metals prior to applying digestate effluent from a recirculating anaerobic digestion system to arable land.

The difference of the microbial community structure and relative abundances between the two reactors (Fig. 7) can be explained by a difference in feeding stratagem. It was previously reported that digestate recycling can heavily influence bacterial communities [30]. As a dominant genus in the recirculated AD reactor for R2, *Candidatus Cloacamonas* is a hydrogen-producing syntrophic bacterium, which is widely present in many anaerobic digesters and constitutes the most dominant taxon at the genus level in AD reactors [31]. As a member of WWE1, *Candidatus Cloacamonas* can utilize several sugars and amino acids and might be involved in cellulose hydrolysis and utilization of fermentation products [32]. *Syntrophus* is an anaerobic bacterium that degrades VFAs and benzoate in *syntrophic* association with hydrogen-using microorganisms [33]. Thus, it shows the more abundant hydrolytic bacteria were existed in R2, operating in digestate recirculation mode. However, the dominant genus in R1 was *Clostridium,* which belongs to the phyla *Firmicutes,* capable of fermenting cellulose and various carbohydrates to acetate, butyrate and hydrogen [34].

The isotope fractionation can be used as an indicator for identifying the predominant methanogenic pathway in anaerobic digesters, which can strongly support molecular analysis [12]. The different $\delta^{13}C_{CO2}$ (‰) and $\delta^{13}C_{CH4}$ (‰) fraction ratios (aC) detected in the present study may have resulted from different predominant methanogenic pathways in R1 and R2 under different experimental phases. Laukenmann et al., 2010 [15] found that methane preferentially hydrogenotrophic methanogens by CO_2 reduction (Eq. 2) with a lower $\delta^{13}C_{CH4}$ (‰) value at higher concentrations of TAN and VFA in

AD plant. This effect may be the reason that $\delta^{13}C_{CH4}$ (‰) in R2 significantly decreased from phase I to II under higher OLRs, which corresponded to higher TAN and VFA concentrations compare with R1. However, $\delta^{13}C_{CH4}$ (‰) in R1 were not significantly different between phase I and II, which may due to the relative stable TAN and VFA concentrations (Fig. 3). The result indicated that hydrogenotrophic may be more dominant in R2 for methanogenesis compare with R1. The decrease in aC values in R1 indicated that acetotrophic methanogens were a more important methanogenesis process in phase II compared with phase I for the control AD reactor, without recirculation. The previous finding supports this hypothesis that at the beginning of incubation, methane was preferentially produced by CO_2 reduction (Eq. 2). Later, methane production by acetate cleavage (Eq. 1) might play a dominant role [35]. However, the aC value in R2 showed contrasting behaviour compared with R1, which increased from phase I to phase II. This result again demonstrated that hydrogenotrophic methanogenesis was the more dominant pathway in R2. Overall, stable isotope analysis results clearly showed an influence of digestate recirculation on methanogenesis pathways in pig manure AD. Nevertheless, continuous monitoring of changing $\delta^{13}C_{CO2}$ (‰) and $\delta^{13}C_{CH4}$ (‰) by stable isotope analysis should be further implemented to evaluate the long-term performance of AD reactors.

5. Conclusions

- The effects of recycled liquid digestate utilization in mesophilic anaerobic digestion of pig manure were investigated in the present study. It was demonstrated that recirculation operation improved methane production and system fermentation stability for OLRs below 5 g VS L^{-1} d^{-1}.
- Long-term recycling of liquid digestate significantly increased viscosity from 30 to 1000 mPa·s, which decreased mass transfer characteristics in AD reactors and further decreased methane production, particularly for an OLR of 6 g VS L^{-1} d^{-1}. The negative effects of accumulated ammonia and VFA on AD treatment of pig manure under

digestate recycling were not found in the present study.

- The liquid digestate recycling intensified the accumulation of heavy metals, including Pb, Mn, Cu and Zn, in both the liquid and solid fractions of the digested effluent, which may impact future application to farmland.
- Stable carbon isotopic analysis of the biogas produced demonstrated different methanogenic pathways between anaerobic reactors with and without liquid digestate recirculation.

Acknowledgements

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Figure 1

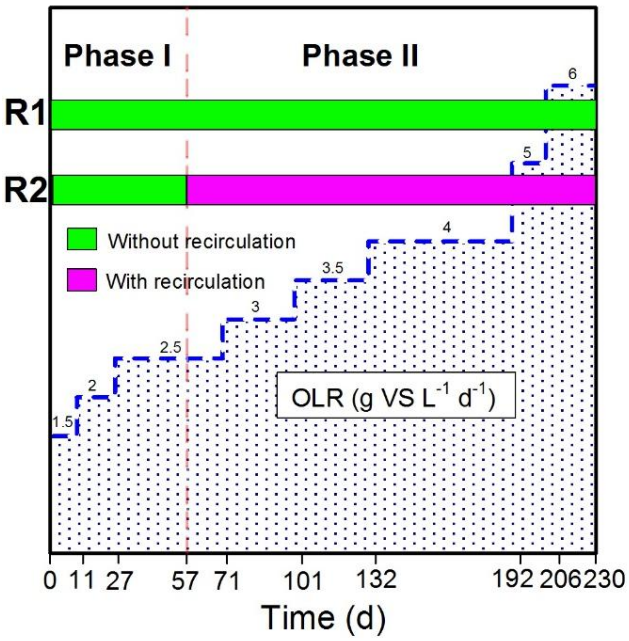

Fig. 1. The operating conditions of the two CSTRs (R1 and R2) for the entire experimental period.

Figure 2

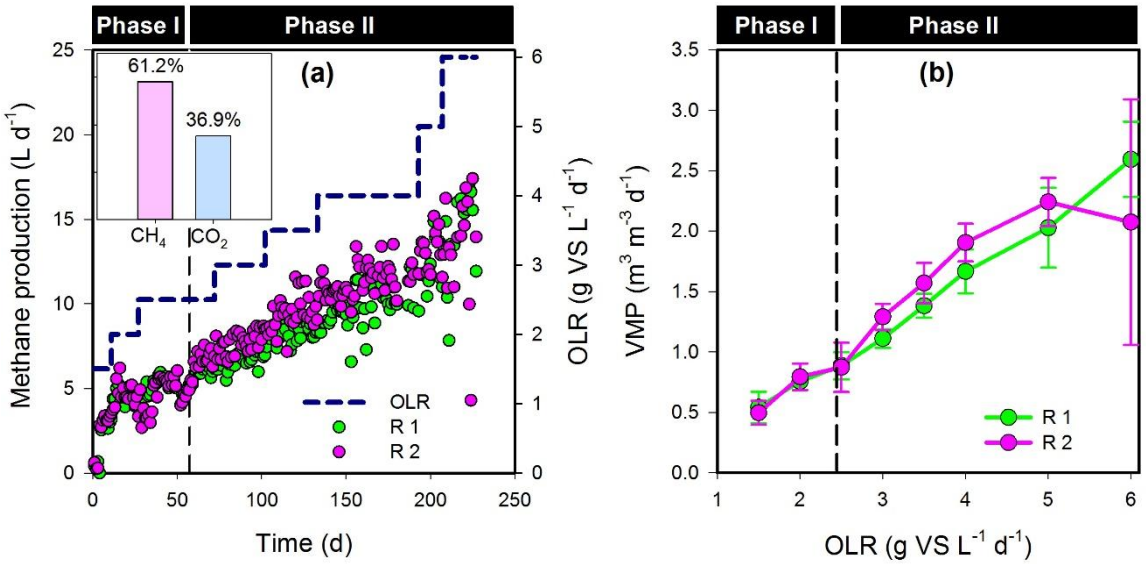

Fig. 2 Dynamics of methane production for the two CSTR reactors (R1 and R2) throughout the experimental phases (a) and the relationship between volumetric methane production (VMP) and organic loading rates (OLRs) (b).

Figure 3

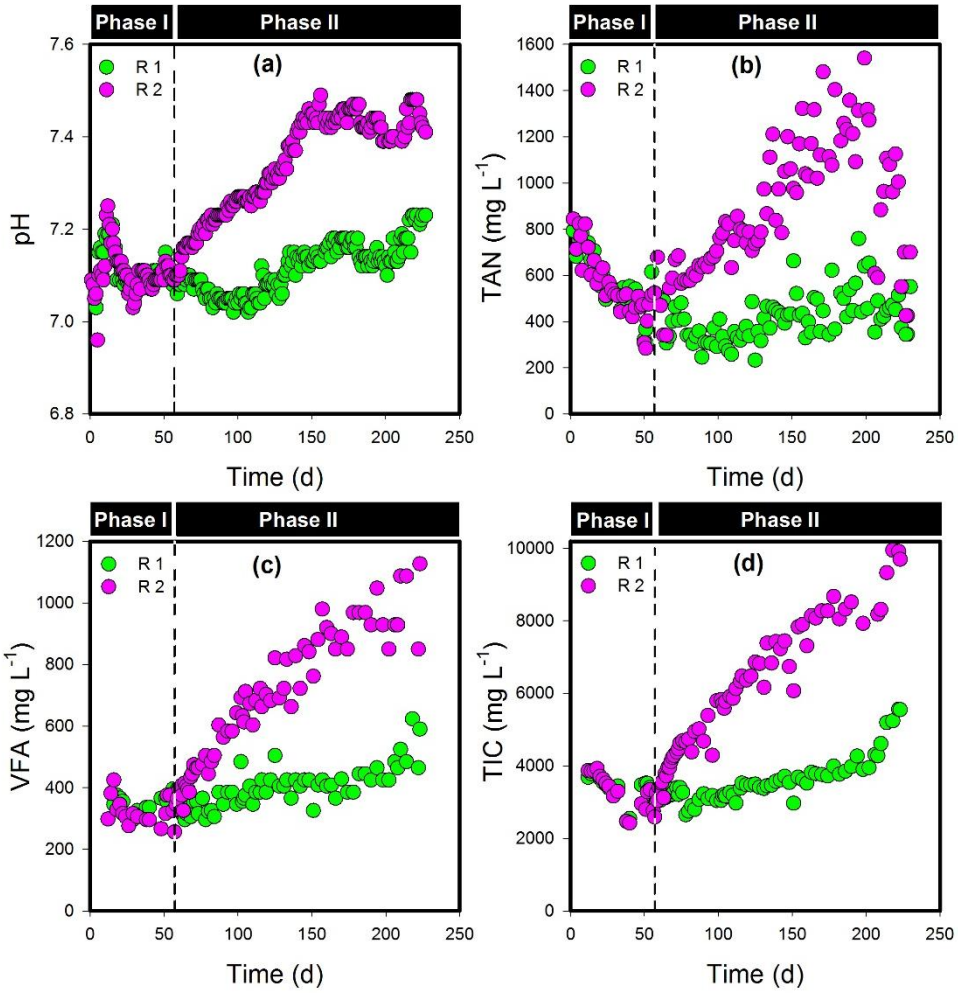

Fig. 3 Dynamics of pH and TAN, VFA and TIC values from the two experimental reactors without (R1) and with (R2) recirculation throughout the experiment.

Figure 4

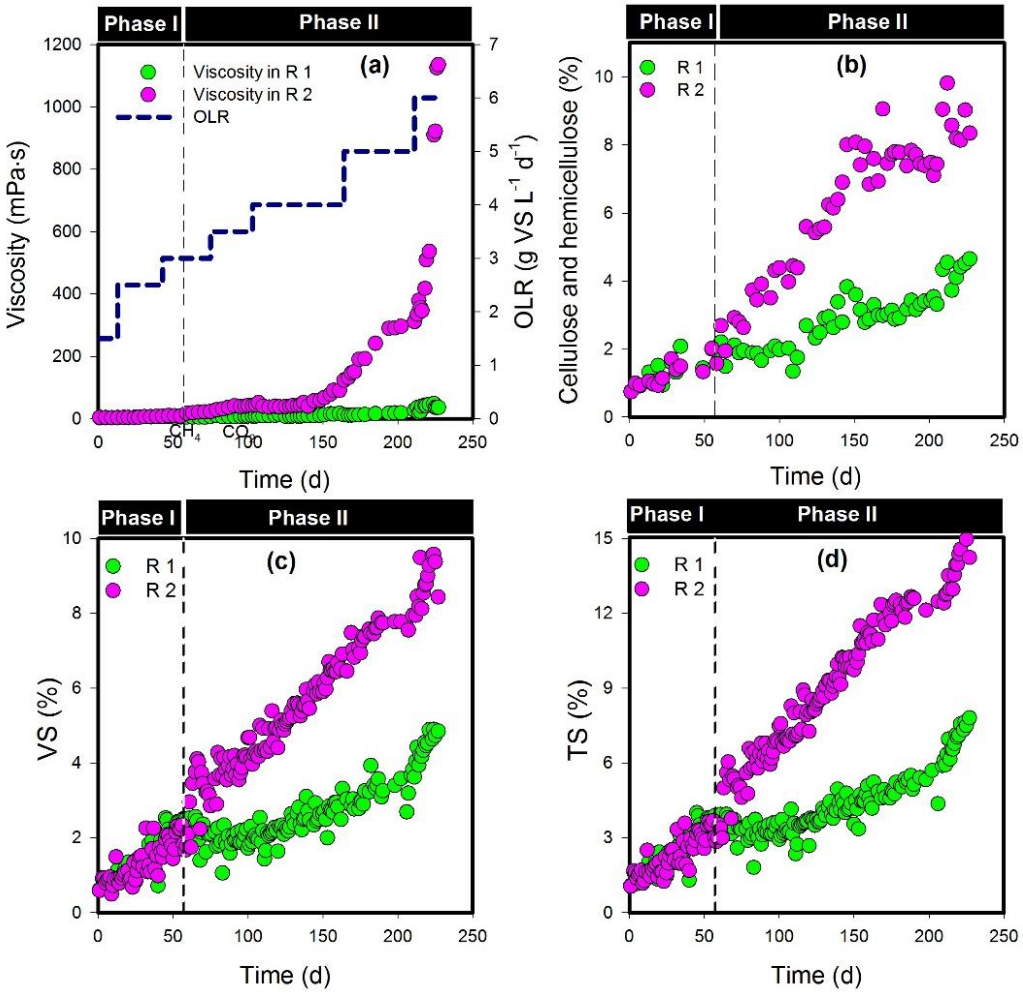

Fig. 4 Dynamics of viscosity, and cellulose and hemicellulose, VS and TS for the two CSTR reactors without (R1) and with (R2) recirculation throughout the experiment.

Figure 5

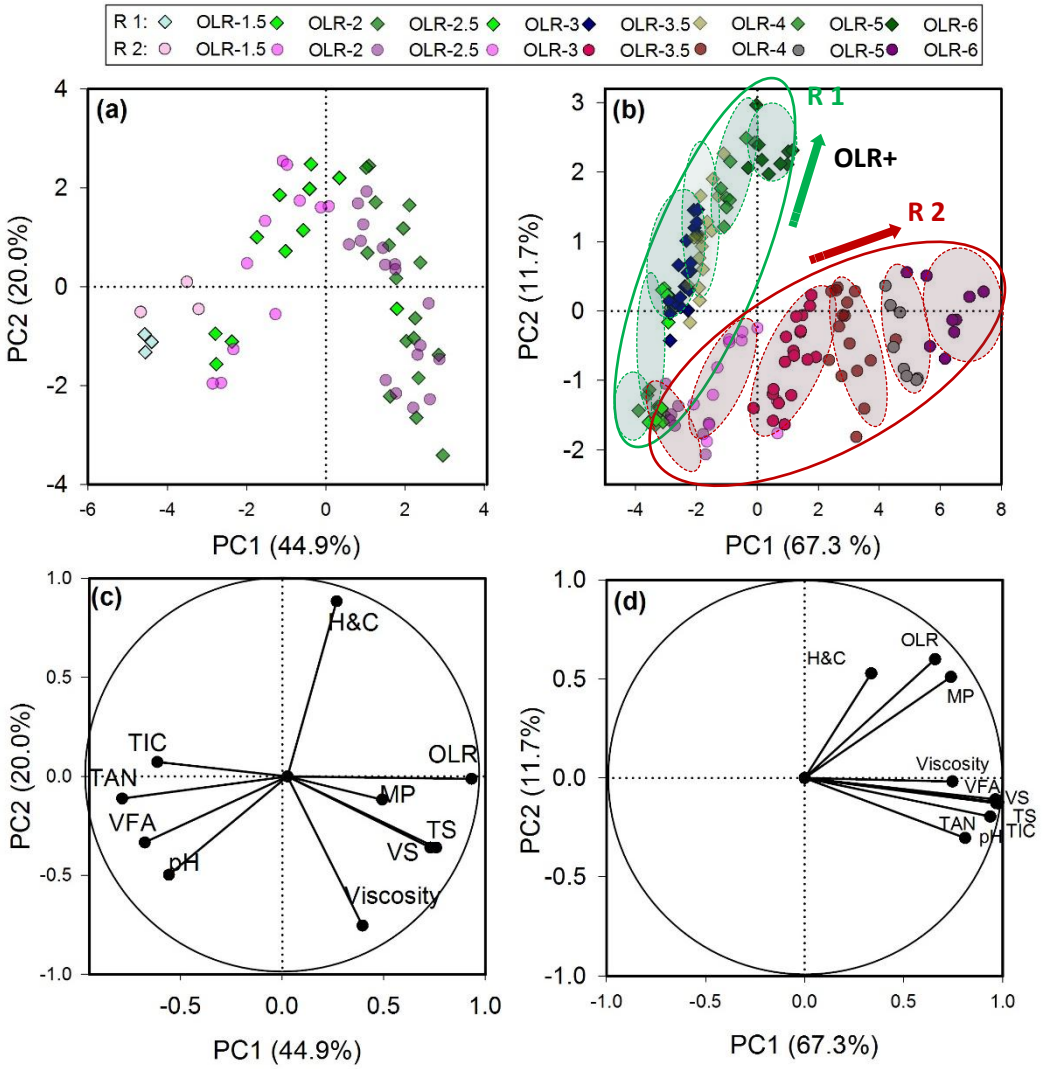

Fig. 5 Principal component analysis of results from the two CSTR reactors without (R1) and with (R2) recirculation in experimental phases I (a) and II (b). Plots (c) and (d) represent the factor loading plots for each principal component analysis in experimental phases I and II, respectively.

Figure 6

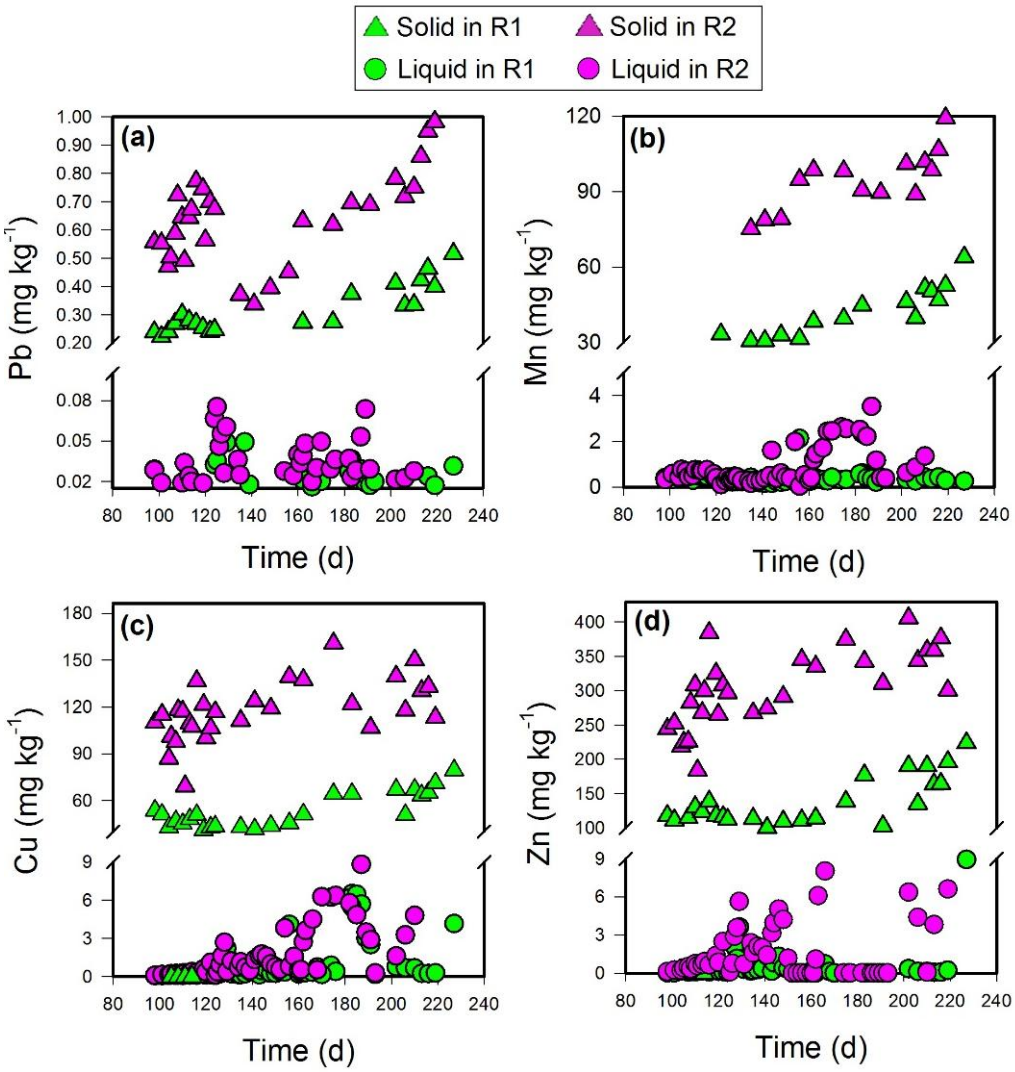

Fig. 6 Dynamics of heavy metal concentrations (Pb, Mn, Cu and Zn) from the two CSTR reactors without (R1) and with (R2) recirculation in experimental phase II.

Figure 7

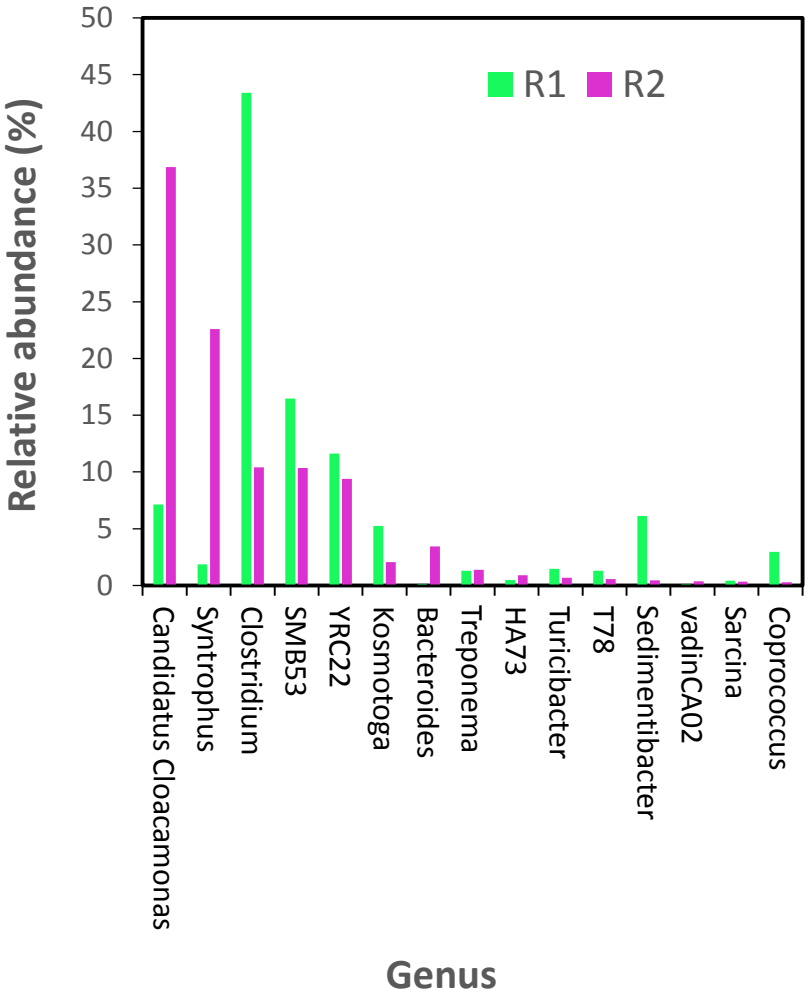

Fig. 7 Taxonomic classification of bacterial 16S rDNA gene reads of the substrate sample from the two CSTR reactors with (R2) and without recirculation (R1) at the genus level (the relative abundances of bacterial 16S rDNA gene reads less than 0.1% are not shown).

Table 1
Characteristics of pig manure and sludge inoculum.

Materials	TS (%)	VS (% TS)	C (% TS)	N (% TS)	H (% TS)	S (% TS)
Pig manure	89.70	68.46	34.38	2.27	5.47	0.58
Sludge inoculum	4.11	54.01	ND	ND	ND	ND

Values are expressed as mean. ND = Not determined.

Table 2

The compositions of $\delta^{13}C_{CO2}$ and $\delta^{13}C_{CH4}$ in the biogas of the two CSTR reactors without (R1) and with (R2) recirculation in experimental phase I (47 d) and II (118 d).

	$\delta^{13}C_{CO2}$ (‰)		$\delta^{13}C_{CH4}$ (‰)		aC	
	Phase I	Phase II	Phase I	Phase II	Phase I	Phase II
R1	5.94 ± 0.45[a]	4.45 ± 0.24[b]*	-44.23 ± 0.91[a]	-44.20 ± 0.63[b]*	1.052 ± 0.005	1.050 ± 0.004*
R2	6.05 ± 0.16	6.00 ± 0.24	-43.90 ± 0.87	-48.21 ± 0.40	1.052 ± 0.003	1.057 ± 0.002

Different letters beside the values from different experiment phases for the same reactor and gas represent significantly different; *beside the values represent significantly different values between different reactors for the same experimental phase and gas.

Supplementary Materials

Liquid digestate recycled utilization in anaerobic digestion of pig manure: Effect on methane production, system stability and heavy metal mobilization

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Table S1

The number of observed valid pyrosequencing reads, OTUs, Shannon-wiener diversity index (Shannon) and Simpson diversity index (Simpson) of the sludge samples from R1 and R2.

Sample	Valid pyrosequencing reads	OTUs	Shannon	Simpson
R1	15,998	840	4.6	0.92
R2	16,134	892	4.1	0.89

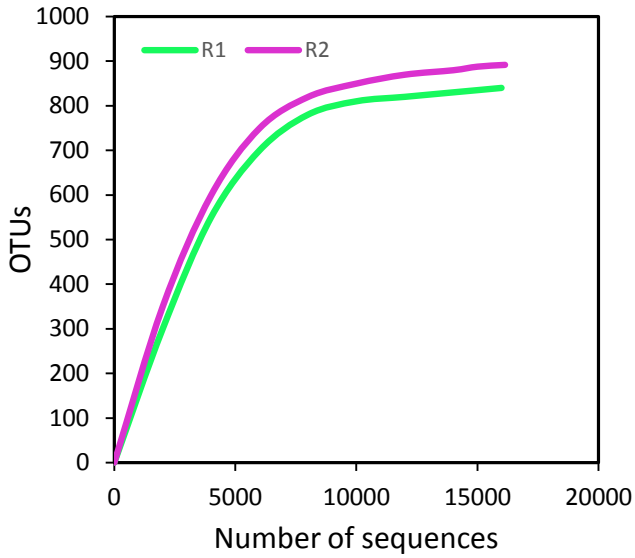

Figure S1. Rarefaction analysis of the sludge samples from R1 and R2. Rarefaction is shown for OTUs with differences that do not exceed 3% OTUs with ⩾97% pairwise sequence identity are assumed to form the same species and genus, respectively.

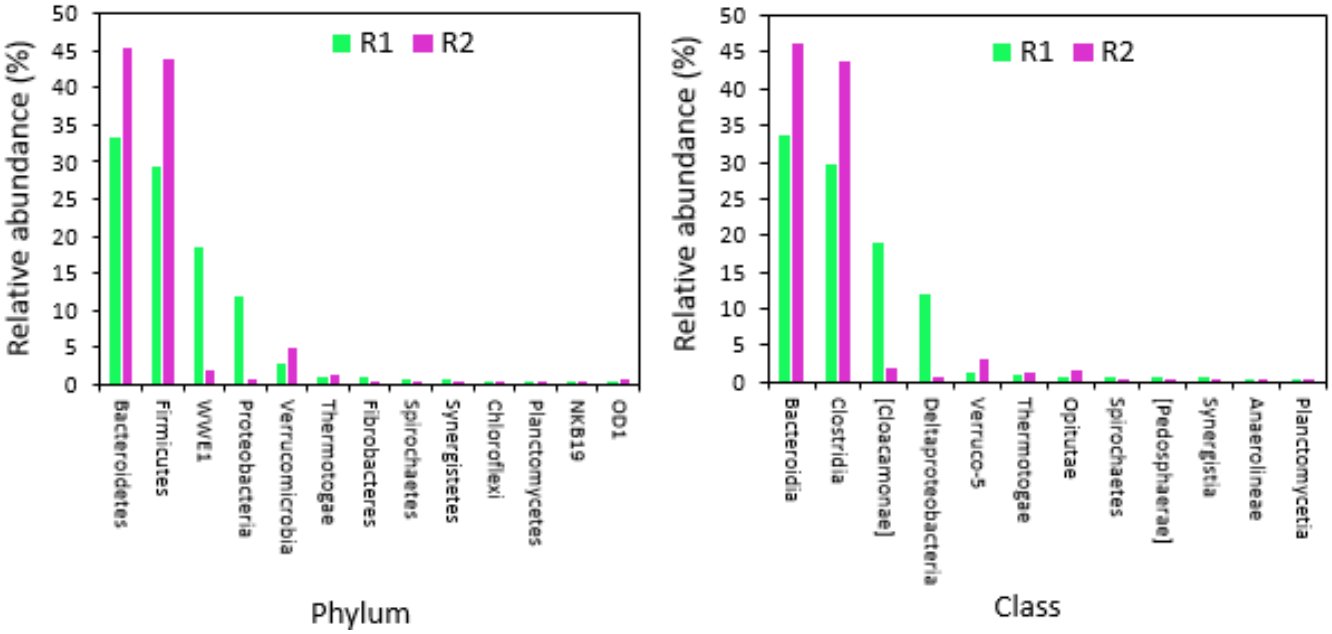

Figure S2. Taxonomic classification of bacterial 16S rDNA gene reads of the substrate sample from two CSTR reactors with (R2) and without recirculation (R1) at phylum and family levels (the relative abundances of bacterial 16S rDNA gene reads less than 0.1% are not shown).