# Methane production in an anaerobic osmotic membrane bioreactor using forward osmosis: Effect of reverse salt flux

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## Abstract

This study investigated the impact of reverse salt flux (RSF) on microbe community and bio-methane production in a simulated fertilizer driven FO-AnMBR system using KCl, $KNO_3$ and $KH_2PO_4$ as draw solutes. Results showed that $KH_2PO_4$ exhibited the lowest RSF in terms of molar concentration 19.1 mM/($m^2$.h), while for KCl and $KNO_3$ it was 32.2 and 120.8 mM/($m^2$.h), respectively. Interestingly, bio-methane production displayed an opposite order with $KH_2PO_4$, followed by KCl and $KNO_3$. Pyrosequencing results revealed the presence of different bacterial communities among the tested fertilizers. Bacterial community of sludge exposed to $KH_2PO_4$ was very similar to that of DI-water and KCl. However, results with $KNO_3$ were different since the denitrifying bacteria were found to have a higher percentage than the sludge with other fertilizers. This study demonstrated that RSF has a negative effect on bio-methane production, probably by influencing the sludge bacterial community via environment modification.

**Keywords***:* Fertilizer, Forward Osmosis, Anaerobic Membrane Bioreactor, Methane, Pyrosequence, Reverse diffusion.

## 1 Introduction

Water scarcity and environmental pollution have driven the development of water reuse

in the urban water management (Shannon et al., 2008). A lot of efforts have been placed to develop technologies to reuse and recycle resource in municipal and industrial wastewater, including activated carbon filtration, ultrafiltration (UF), nanofiltration (NF), reverse osmosis (RO) and anaerobic membrane bioreactor (AnMBR) (Michael et al., 2013; Wei et al., 2014). Although these technologies showed a good capability to remove suspended solids, organic pollutants, and even salinity from the wastewater, there are still some challenges to be addressed. For instance, the emerging micro pollutants were difficult to be completely removed by AnMBR equipped with UF membrane (Cath et al., 2006). On the other hand, RO is an energy intensive treatment technology, which cannot be affordable in some developing countries where coincidently water demand is also high, such as in the Middle East and North Africa (MENA) (Ghaffour, 2009). It is therefore crucial to develop a novel technology capable of removing emerging micro pollutants at low energy cost.

Forward osmosis (FO) is a membrane separation process driven by the osmotic pressure difference between the feed solution (FS) and draw solution (DS) (Cath et al., 2006). Since this process is not driven by an external pressure, the energy consumption is much lower than RO technology. It has been reported that the fouling and scaling of FO membranes is also less severe than in RO, and mostly reversible via hydraulic cleaning (Li et al., 2015). However, FO generally needs to be coupled with another process to separate the diluted draw solution from the final product water. However, this additional recovery process requires energy and increases the capital cost of the hybrid system . Lately, fertilizer-drawn forward osmosis (FDFO) has received increased interest since the diluted DS can be used directly for irrigation purposes and therefore no recovery process is required (Phuntsho et al., 2012). In FDFO, the process is driven by fertilizers (FO draw solution) and thus the water drawn from the wastewater (FO feed solution) is used to dilute the fertilizer solution which can then be directly used for fertigation (Chekli et al., 2017; Kim et al., 2016).

By combining FDFO and AnMBR, several benefits can be achieved, namely bio-methane production, higher effluent quality than UF based AnMBR systems and sustainable fertigation via wastewater reuse. Different fertilizers have been compared in terms of water flux, bio-methane production rate and reverse diffusion (Kim et al. 2016).

However, it is still not clear why different fertilizers exhibited different bio-methane production rates. It could be related to the bacterial community variation caused by different reverse diffusion rates of different fertilizers, but there is no substantial proof to support this hypothesis.

Due to the RSF from the fertilizer draw solution (DS) and the high salt rejection of the FO membrane which retains the salts from the feed, the fertilizer concentration within the AnMBR will ultimately increase. However, this increase is a gradual process rather than a one-time intensive dosage. The bacterial community in the AnMBR is sensitive to the environment, especially the methanogens, so the one-time intensive dosage of fertilizer could significantly modify the bacterial community but might not reflect the real situation with gradual increase. Since the methanogens might be able to endure the gradual changes of environment but not a sudden significant modification, the one-time intensive fertilizer dosage may overestimate the effect of reverse diffused fertilizers on the methane production in the FDFO-AnMBR. Therefore, a systematic study is required to demonstrate the impact of RSFs of different fertilizer DS on bio-methane production in the FDFO-AnMBR, especially in a parallel comparison with gradual build-up of salt.

This study investigated the impact of gradual reverse fertilizer diffusion on the methane production in a hybrid FDFO-AnMBR system by dosing three different fertilizers, which amount was pre-determined via FO experiments, into parallel anaerobic fermentation bottles step by step. The methane production was monitored for all conditions to check the effects of fertilizer dosage. The corresponding sludge under different conditions were also collected and analyzed via pyrosequencing to illustrate the microbe community difference of different conditions and its relation with the methane production difference.

# 2 Materials and Methods

## 2.1 Anaerobic sludge

The anaerobic sludge collected from one digester of the Wollongong Sewage Treatment Plant, located in Wollongong, Australia (Lat: 34 26 35 S Long: 150 53 50 E), was used as the seed sludge in this study. 700 mL of anaerobic sludge was filled in each bottle of the bio-methane potential (BMP) apparatus and then purged by nitrogen gas to ensure the anaerobic condition within these bottles to simulate AnMBR systems. The anaerobic

sludge was characterized in terms of total solids (TS), mixed liquor suspended solids (MLSS), pH and chemical oxygen demand (COD) (Table 1).

## 2.2 Model substrates and reversed draw solutes

To maintain the bioactivity of the BMP apparatus, 550 mg/L glucose was dosed in the bottles every two day as substrate for the anaerobic fermentation. Glucose is a common compound utilized as model substrates in membrane bioreactor research (Ansari et al., 2015). The amount of glucose dosed every two day in this study was determined based on the synthetic wastewater recipe used in a previous study (Kim et al., 2016) assuming 1 L of treated wastewater per day.

Three fertilizers, namely KCl, $KNO_3$ and $KH_2PO_4$ were used as model draw solutes in the FDFO process for this study. These three fertilizers were chosen because they exhibited different RSF according to preliminary results (S. Li et al., 2017), and thus the impact of reversely diffused fertilizers on the bio-methane production could be evaluated. The experiment was conducted for 20 days, since one similar study reported that the methane production trend and microbial community dynamics in an anaerobic digester did not change after 20 days (Wang et al., 2017). In contrast with one time intensive dosage of fertilizer in a previous reported study (Kim et al., 2016), the fertilizers were gradually added in the fermentation bottles over the whole experiment period of 20 days. To simplify the simulation, the RSF was considered constant during the whole experiment of 20 days. The amount of dosed fertilizer per day was based on the detected RSF (described in Section 2.3), membrane area of 20 $cm^2$ and a 24-hour operation.

## 2.3 FO experiments for RSF determination

To determine the amount of fertilizer chemicals to be added to the digested sludge, FO experiments were conducted to evaluate the RSF of different draw solutes (Supplemental Table S1 and Figure S1 for experimental conditions and schematic diagram, respectively). During these RSF determination experiments, primary wastewater and corresponding fertilizers were utilized as FS and DS, respectively. The FS and DS of 1 L each were separately recycled in the FO system with an identical velocity of 8.5 cm/S (Table S1). The experiment was conducted for 24 hours, the volume of DS increased by

240 mL for all three tested fertilizer DS. The FS and DS before and after experiment were sampled for $NO_3^-$ and $PO_4^{3-}$ analyses with ion chromatography system (ICS-1600, DIONEX) and $K^+$ analyses with inductively coupled plasma – mass spectrometry (ICP-MS, Agilent Technology 7500 series). The RSF was determined based on Eq. 1:

$$RSF = (C_{F\_end} \times V_{F\_end} - C_{F_{ini}} \times V_{F_{ini}})/(A \times T) \quad \text{Eq. (1)}$$

where $C_{F\_ini}$ and $C_{F\_end}$ are the draw solute concentration in the FS at the beginning and the end of the FO process, and $V_{F\_ini}$ and $V_{F\_end}$ are the volume of FS at the beginning and the end of FO process. $A$ is the effective FO membrane area used in this study (20 $cm^2$), and $T$ is the time of the FO experiment (24 hours).

As shown in Figure S2, the detected RSFs for $KH_2PO_4$, KCl and $KNO_3$ and draw solutes were 2.6, 2.4 and 12.2 g/($m^2$.h), corresponding to 19.1, 32.2 and 120.8 mM/($m^2$.h), respectively. Therefore, in terms of RSF in molar concentration, $KH_2PO_4$ had the lowest reverse diffusion among the three tested fertilizers.

## 2.4 Bio-methane potential (BMP) apparatus

Because the bacterial community could change over time, in order to investigate the effect of different RSFs under identical initial conditions, the bio-methane potential experiments were conducted in a batch mode for different substrates addition (Ansari et al., 2015) using a BMP apparatus (depicted in Figure S3). The BMP apparatus is composed of 7 fermentation bottles submerged in a water bath at a temperature of 35±1°C. The generated biogas from these bottles was diverted to an array of inverted 1,000 mL plastic mass cylinders submerged in the 1 M NaOH solution to collect and measure the biogas. The NaOH solution plays an important role to remove $CO_2$ and $H_2S$ from biogas to better evaluate $CH_4$ production potential. Air volume in each mass cylinder was recorded 2 times per day. Detailed description of BMP apparatus used in this study is given elsewhere (Ansari et al., 2015).

## 2.5 Experimental protocol

First of all, the quality of anaerobic sludge was characterized and described in Section 2.1. The RSFs of FDFO process was determined for three different fertilizers and then the corresponding amount of each fertilizer was added into the BMP apparatus together with

glucose for the bacteria growth during anaerobic sludge fermentation. One fermentation bottle was filled with DI water as blank control, while the rest six bottles were filled with fertilizers (each fertilizer was run in duplicate). After mixing the anaerobic sludge with the substrates and fertilizers, all bottles were purged with nitrogen gas. The dissolved oxygen in fermentation bottles was measured after nitrogen gas purging to ensure the value was lower than 0.5 mg/L. The fermentation bottles were then submerged in a water bath of 35°C and connected to the biogas collecting cylinder described in Section 2.5. During fermentation, glucose was added into the anaerobic sludge every two days as indicated in Section 2.2. On the other hand, the corresponding amounts of different reverse diffused fertilizers determined in Section 2.3 were dosed every three days to simulate the gradual accumulation in an AnMBR system. The produced biogas volume was continuously recorded, and the methane, nitrogen concentration within the collected biogas was determined after the experiment. Moreover, the corresponding anaerobic sludge under different fertilizer addition was collected for bacteria and Achaea composition investigation via pyrosequencing.

## 2.6 Bio-methane determination

The biogas in each cylinder was collected with a 1-liter gas-sampling bag. After the collection, the volumes of bio-methane, nitrogen and carbon dioxide were determined by a portable methane detection apparatus (Multitec 560, Orangeth). The specific biogas volume was calculated based on the Eq. (2).

$$V_s = C_m \times V_b \quad \text{Eq. (2)}$$

where $V_s$ is the produced specific biogas volume, $C_m$ is the measured percentage of specific biogas, and $V_b$ is the recorded volume of biogas mixture.

## 2.7 DNA extraction

The sludge samples collected at the end of the experiment from the bottles of the BMP apparatus were stored under -20°C before shipping to DNASense Apps Company in Denmark for 454 pyro-sequencing. During the shipment, sludge samples were kept in dry ice at a temperature of -20°C, as recommended. The DNA of all bacteria and archaea in sludge samples were extracted through the FastDNA Spin kit for soil (MP Biomedicals,

USA), using 4x the normal bead beating to enable the recovery of bacteria that are difficult to lyse (Albertsen et al., 2015).

## 2.8 16S rRNA amplicon library preparation

The procedures for bacterial and archaeal 16S rRNA amplicon sequencing were based on (Caporaso et al., 2012) and an Illumina protocol (Illumina, 2015), respectively. Up to ten ng of extracted DNA was used as template for PCR amplification of the 16S rRNA gene fragments. Each PCR reaction (25 µL) contained dNTPs (400 µM of each), MgSO4 (1.5 mM), Platinum® Taq DNA polymerase HF (0.2 mU and 0.5 U for bacteria and archaea, respectively), 1X Platinum® High Fidelity buffer (Thermo Fisher Scientific, USA) and tailed primermix (400 nM of each forward and reverse). The forward and reverse tailed primers were designed (Illumina, 2015) and contained primer parts targeting the respective 16S gene fragments. For bacterial community, V1-3 primer containing 27F AGAGTTTGATCCTGGCTCAG and 534R ATTACCGCGGCTGCTGG was used; while V3-5 primer with 5'-CCCTAHGGGGYGCASCA (Arch-340F) and 5'-GWGCYCCCCCGYCAATTC (Arch-915R) was used for archaea. PCR was run with the following programs: 1) bacteria: initial denaturation at 95°C for 2 min, 30 cycles of amplication (95°C for 20 s, 56°C for 30 s, 72°C 60s) and a final elongation at 72°C for 5 min; 2) archaea: initial denaturation at 95°C for 2 min, 35 cycles of amplication (95°C for 20 s, 50°C for 30 s, 72°C 60s) and a final elongation at 72°C for 5 min. Duplicate PCR reactions were performed for each sample and duplicates were pooled after PCR. Both bacteria and archaea amplicon libraries were purified using the Agencourt® AMpure XP bead protocol (Beckmann Coulter, USA) with the following exceptions: the sample/bead solution ratio was 5/4, and the purified DNA was eluted in 33 µL nuclease-free water. Library concentration was measured with Quant-iT$^{TM}$ HS DNA Assay (Thermo Fisher Scientific, USA) and quality validated with a Tapestation 2,200, using D1K ScreenTapes (Agilent, USA).

The purified sequencing libraries were pooled in equimolar concentrations and diluted to 4 nM. The samples were paired end sequenced (2×301 bp) on a MiSeq (Illumina) using a MiSeq Reagent kit v3, 600 cycles (Illumina, USA) following the standard guidelines for preparing and loading samples on the MiSeq. 20% Phix control library was spiked in to

overcome low complexity issue often observed with amplicon samples.

The DNA extraction and sequencing were successful for all samples and yielded 28,720-149,886 and over 10000 reads for bacterial and archaeal 16S rRNA genes, respectively, after quality control and bioinformatics processing.

## 2.9 Bioinformatics analysis

Forward and reverse reads for both bacteria and archaea were trimmed for quality using Trimmomatic v. 0.32 (Bolger et al., 2014) with the settings SLIDINGWINDOW:5:3 and MINLEN:275. Because the V3-5 region for archaea is longer than what is possible to merge, only the first 275 bp of read 1 was used for further analysis. Both bacteria and archaea reads were dereplicated and formatted for use in UPARSE workflow (Edgar, 2013). The dereplicated reads were clustered, using the usearch v. 7.0.1090 -cluster_otus command with default settings. OTU abundances were estimated using the usearch v. 7.0.1090 –usearch_global command with –id 0.97. Taxnomy was assigned using the RDP classifier (Wang et al., 2007) as implemented in the parallel_assign_taxonomy_rdp.py script in QIIME (Caporaso et al., 2010), using the MiDAS database v.1.20 (McIlroy et al., 2015). The results were analyzed in R (RCoreTeam, 2015) through the Rstudio IDE using the ampvis package v.1.9.1 (Albertsen et al., 2015).

## 2.10 Acetate analysis

To support the possible abundance of acetogenesis bacteria in sludge, acetate (output of acetogenesis bacteria) as an indicator was measured. Samples of sludge with different fertilizer addition (10 mL for each sample) were collected during the experiments, and stored at -20°C. The acetate concentrations of samples were determined with acetate colorimetric assay kit following the instruction procedure (MAK086, Sigma-Aldrich).

# 3 Results and discussion

## 3.1 Methane production in anaerobic fermenters

Biogas production from the anaerobic digestion of fermented anaerobic sludge is shown in Figure 1 for different fertilizer dosages. With the increase of fermentation time, more

biogas was produced for all conditions, and the amount of produced biogas was similar for both sludge without fertilizer (DI), and sludge with $KNO_3$ and $KH_2PO_4$ dosages. However, the biogas production from sludge with KCl dosage exhibited a slightly lower total biogas volume than others. Total biogas volume is composed of methane and nitrogen. The dosage of fertilizer could affect the archaea and bacteria genus responsible for methane and nitrogen production, and eventually led to the variation of total biogas volume. Therefore, the slight difference in total biogas production of fermenters with KCl dosage could be due to the bacteria and archaea community difference between different fertilizer dosages.

Although the biogas production was similar to all conditions, the methane and nitrogen concentrations exhibited a significant variation for different conditions. As shown in Figure 2, around 272 mL produced biogas was methane when there was no fertilizer in the sludge (DI), while the methane volume within biogas for sludge with fertilizers was lower and varied with the type of fertilizer used. In general, the higher RSF was, the less methane was produced. $KH_2PO_4$ dosage exhibited the closest methane volume to the DI condition (238 mL), followed by KCl dosage (170 mL) and $KNO_3$ dosage (less than 65 mL). Interestingly, RSFs of these three fertilizers followed a reverse order. Moreover, the sludge with $KNO_3$ exhibited a higher nitrogen gas concentration (1,166 mL) than the other conditions which were between 820 mL and 990 mL. Since the dosages of fertilizers were based on simulated FDFO processes, the impact of different fertilizers' RSF on the anaerobic bio-methane production can thus be demonstrated. Fertilizers' effect on changing the biogas production and composition could be due to: 1) the acute responses of sludge in the initial period due to different fertilizer dosages; 2) and further variations of microbe communities in the long-term. It has been reported that the increase of ionic strength could inhibit the viability of microbes (Cha et al., 2013). With the increase of RSF and feed solute rejection, the ionic strength of sludge would also increase which could also contribute towards inhibition of microbes. However, because the increase of salinity is gradual, the negative influence of increase in salt concentration (such as $KH_2PO_4$) on bio-methane production at the initial period might not be as severe as reported in the studies since the microbe communities have time to adapt to the new conditions (Ansari et al., 2015; Kim et al., 2016).

Regarding the variations of microbe communities, both archaea and bacteria might involve. Methane production involves hydrolysis, acidogenesis, acetogenesis and methanogenesis (Chojnacka et al., 2015). Archaea is mainly responsible for the final stage of methanogenesis, while the previous steps are conducted by the bacteria. Both archaea and bacteria have different types of genus involving the whole process of methane production via different pathway. Hydrogenotrophic methanogens mainly utilize $CO_2$, $H_2$ and formate as substrates for methane production while acetotrophic methanogens utilize acetate and methylotrophic methanogens utilize methylamines (Chojnacka et al., 2015). Bacteria in anaerobic sludge mainly contribute to the hydrolysis and biodegradation of the organics into substrates utilizable for methanogens, such as $CO_2$, $H_2$, formate, propionate and acetate. All these archaea and bacteria genus have their own optimum growth environment. The reverse diffusion of fertilizer could therefore substantially change the environment for bacteria and archaea. Besides the inhibition of microbes' viability in the initial period, the microbial community could also be changed in long-term. Consequently, the amount of archaea and bacteria for methane production could be directly/indirectly affected. Some archaea are sensitive to the salinity, such as *Methanomethylovorans* (Cha et al., 2013); thus its viability might be firstly inhibited and then followed by a reduction in amount due to the addition of fertilizers; while some bacteria can grow better in a mineral medium (e.g., *Enterococcus* (Fisher & Phillips, 2009) and *Comamonas* (Etchebehere et al., 2001)) which could help them outcompete other bacteria genus when the salinity of sludge is increased by the fertilizer addition. The increase of nitrogen gas for sludge with $KNO_3$ addition was probably related to the abundance of de-nitrification bacterium, *Comamonas,* which could convert the reverse diffused nitrate into nitrogen.

One previous study about the selection of FDFO draw solutes showed that the biomethane production was significantly affected by KCl, $KH_2PO_4$, $(NH_4)_2SO_4$, $NH_4Cl$, $NH_4NO_3$, except for $(NH_4)_2HPO_4$ (Kim et al., 2016). In contrast with this study, the dosage of KCl, $KH_2PO_4$ and $KNO_3$ exhibited a clear difference in terms of biomethane production. That is probably because high fertilizer dosages (9 times concentrated) were applied in the previous study (Kim et al., 2016), which might have exposed the archaea and bacteria in sludge to a mineral shock and severely affect the viability of microbial

communities and thus no methane was produced in most of the conditions tested. However, in the present study, the fertilizers were gradually added into the sludge and this procedure gave some time to the microbes to adjust themselves for the change of environment. Considering that the concentration of fertilizer in anaerobic digester is also a gradual process, the step-by-step dosage of fertilizer can better reflect the impact of reverse fertilizers on the biomethane production.

## 3.2 Effect of fertilizer types on the archaeal community structure of anaerobic sludge

In Figure 3, the overall microbial compositions in all samples are compared using multivariate statistics, principal components analysis (PCA), in which samples located close to each other imply a similar microbial composition. As shown in the figure, the duplicates of KCl and $KNO_3$ are located next to each other (except for $KH_2PO_4$), indicating that the microbial compositions in the duplicates of these two conditions were very similar. The reason behind the slight different archaeal composition for duplicates of $KH_2PO_4$ was not clear. Since all the experimental conditions and seed sludge were identical for the two duplicates of $KH_2PO_4$ during the experiments, the slight composition difference could be due to slight manual operational variation during sludge sampling, sample shipment and DNA extraction procedure. However, although there are differences between the microbial compositions with different fertilizers, all samples clustered in a small area, indicating that the archaea composition of all samples is similar to each other in general.

All the detected archaea are within the *Eurychaeota* phylum. The *Euryarchaeota* include the methanogens producing methane and often found in intestines, the halobacteria surviving at extreme concentrations of salt, and some extremely thermophilic aerobes and anaerobes (Amils, 2011). The genus of abundant archaea under all tested conditions is shown in Figure 4. This includes *Methanosaeta, Methanobacterium, Methanomethylovorans, Methanobrevibacter, o_WCHA1-57_OTU_9, Methanospirillum, Methanomassiliicoccus, f_WCHA2-08_OTU_13, Methanoculleus, Methanosphaera*. As shown in this figure, the dominant archaea genus for all conditions is *Methanosaeta*. The relative abundance of *Methanosaeta* for DI, KCl, $KNO_3$ and $KH_2PO_4$ was 98.1%, 98.2%,

95.2% and 97.8%, respectively. The rest of archaea only covered 2-5% of the abundance. This result is in good agreement and supports the similar microbial composition observed in Figure 3.

*Methanosaeta* are gram-negative rods typically 0.8 - 1.3 by 2 - 6 μm in size (Kamagata et al., 1992). They are commonly found in rice paddies and anaerobic digesters which are common sources of methane (Smith & Ingram-Smith, 2007). *Methanosaeta* are acetotrophic methanogen, which means they rely on the acetate for methane production during anaerobic digestion (Mori et al., 2012). These organisms are widely distributed across the planet, and have an extremely high affinity for acetate allowing them to thrive even if concentrations are very low (5-20 μM) (Jetten et al., 1992). Among the methanogens detected in this study, *Methanosaeta* is the only acetotrophic methanogen while the others are hydrogentrophic methanogens that obtain energy for growth by using hydrogen to reduce $CO_2$ to $CH_4$.

The occurrence of *Methanosaeta* in the anaerobic digester was very likely because of the low acetate concentration (<45 μM for all fertilizer conditions, Figure S4). In fact, *Methanosaeta* dominate in stable habitats, where acetate levels are low, since they are specialists with a higher affinity for acetate (Jetten et al., 1990). The acetate concentrations in all digesters are generally within the threshold range for *Methanosaeta* growth (7-70 μM), but not sufficient for the growth of other acetotrophic methanogen, such as *Methanosarcina* (0.2-1.2 mM required) and hydrogentrophic methanogens (0.4-0.6 mM required) (Jetten et al., 1990). On the other hand, the dominant abundance of *Methanosaeta* (more than 95% for all conditions) also indicated that acetate in sludge was crucial for the methane production in this study. Therefore, the methane production in such a *Methanosaeta* dominating system depended almost entirely on the abundance of acetogenesis bacteria. The difference of biomethane production observed in this study was therefore very likely caused by the variation of bacteria that could affect acetogenesis.

## 3.3 Effect of fertilizer types on the bacterial structure of anaerobic sludge

The bacterial composition of different conditions is presented in Figure 5. The duplicates

of each tested fertilizer are located close to each other in the figure, confirming the similarity of the bacterial composition between two duplicates and the reproducibility of experiments. It is also clear that the bacterial composition of $KH_2PO_4$ exhibited the highest similarity to the DI condition without fertilizer. Bacteria composition with KCl addition exhibited a higher difference than $KH_2PO_4$, while bacterial composition with $KNO_3$ addition is located far from the DI condition and other two fertilizers; exhibiting the highest difference. It is interesting to note that the distances between bacteria compositions of different fertilizers and DI condition (shown in Figure 5) are consistent with the corresponding differences in bio-methane production (Figure 2) and the amount of fertilizer addition determined by the FO process. The more similar bacterial composition between fertilizer addition and DI condition was, the more bio-methane would be produced. This confirms that the reverse diffusion of fertilizers affected the bacteria composition by changing their living environment, and thus influencing the bio-methane production.

40 bacteria genus were detected to have more than 0.1% relative abundance in all samples (Figure S5). Within these 40 genus, 8 bacteria genus were found to have different percentage for different fertilizer conditions. Figure 6 exhibits the bacteria genus variation under different conditions, providing proofs to elucidate the impacts of bacteria composition on bio-methane production. The 8 genus include *Enterococcus* and *Trichococcus* of *Firmicutes* phylum, *vadinBC27 wastewater-sludge group*, *Proteiniphilum* and *f_009E01-B-SD-P15_OTU_19* of *Bacteroidetes* phylum; *Syntrophorhadus* and *Comamonas* of *Proteobacteria* phylum; *f_Spirochaetaceae_otu_58* of *Spirochaetae* phylum. Within these genus, *Trichococcus* (Regueiro et al., 2014), *Proteiniphilum* (Chen & Dong, 2005), *f_009E01-B-SD-P15_OTU_19, Syntrophorhadus* (Ju & Zhang, 2014) and *f_Spirochaetaceae_otu_58* are acetogenesis bacteria, capable of biodegrading organics into acetate. Sludge without fertilizer (DI condition) showed similar percentages on all genus to sludge with $KH_2PO_4$, confirming the similar bacteria composition of these two conditions.

Considering that the acetotrophic *Methanosaeta* is the dominant archaea for all conditions, it indicates that the similar methane production for these two conditions was likely because the simulated $KH_2PO_4$ reverse diffusion did not cause significant changes

on the sludge microbial composition. Since the reverse diffusion of $KH_2PO_4$ was the lowest among the three tested fertilizers, it indicates that the reduction of methane production in FO-AnMBR could be minimized by limiting the reverse draw solute diffusion. Regarding the sludge with KCl addition, the abundance percentages of most genus were similar to the DI condition, except for the *Enterococcus* and *Trichococcus*. *Enterococcus* is Lactic acid production bacteria (Fisher & Phillips, 2009). The *Enterococcus* was higher in KCl condition than DI and $KH_2PO_4$, which could produce more lactic acid instead of acetate, which might not be suitable for the consumption of *Methanosaeta*. Moreover, the lower abundance of *Trichococcus* in KCl and $KNO_3$ conditions could lead to an even lower acetate production in the digester and thus a lower methane production, and this has been confirmed by the methane production shown in Figure 2. Sludge with $KNO_3$ exhibited the lowest methane production, and interestingly, besides the lower abundance of *Trichococcus*, the abundances of all other acetogenesis bacteria in this condition were lower than other three conditions. Moreover, there were three bacteria genus in sludge with $KNO_3$ which exhibited higher abundance percentages than other conditions, which includes *Enterococcus*, *vadinBC27 wastewater-sludge group* and *Comamonas.* As mentioned above, *Enterococcus* produce lactic acid, not acetate, during anaerobic digestion and this might lead to the lower methane production. *Comamonas* is an anoxic denitrifier (Chen et al., 2016; Etchebehere et al., 2001), so it could utilize the nitrate in sludge as electron accepter and produce nitrogen gas, and consequently outcompete other acetogenesis bacteria and indirectly reduce the methane production. This can be confirmed by the higher detected nitrogen gas production in sludge with $KNO_3$ addition. Another possibility could be that the nitrate is toxic for the acetogenesis bacteria and thus, the reverse diffusion of nitrate from $KNO_3$ led to the reduced abundances of these bacteria genus.

## 4 Conclusions

This study demonstrated the impact of fertilizers' RSF on methane production in a hybrid FDFO-AnMBR system and the mechanisms related to microbe composition. Different fertilizers exhibited different RSFs with an order of $KH_2PO_4$<KCl<$KNO_3$, and therefore different negative impacts on the bio-methane production. The impact of RSF on methane

production was found to be through changes on the bacterial community in the AnMBR system, instead of the archaea community. The lowest methane production observed for $KNO_3$ fertilizer was also probably due to the promoted denitrification bacteria abundance caused by the elevated nitrate concentration, which in turns outcompeted the acetogenesis methanogens.

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**Figure captions**

**Figure 1:** Accumulated produced biogas volumes within anaerobic fermenters with different reverse diffused fertilizers during a 20-day experiment. RSFs for $KH_2PO_4$, KCl and $KNO_3$ and draw solutes were 2.6, 2.4 and 12.2 g/($m^2$.h), corresponding to 19.1, 32.2 and 120.8 mM/($m^2$.h), respectively (Fig. S2). The values shown in the Figure are the averages of duplicate experiments using each different fertilizer dosage, except for the blank control with DI water. Complete composition of accumulated produced biogas is shown in Table S2.

**Figure 2:** Methane ($CH_4$) and Nitrogen ($N_2$) gas volume in the accumulated produced biogas for anaerobic fermenters with different reverse diffused fertilizers. RSFs for $KH_2PO_4$, KCl and $KNO_3$ and draw solutes were 2.6, 2.4 and 12.2 g/($m^2$.h), corresponding to 19.1, 32.2 and 120.8 mM/($m^2$.h), respectively (Fig. S2).

**Figure 3:** Principle component analysis of archaeal community in sludge samples. Each point represents the microbial community in a specific sample. Distance between the sample dots signifies similarity; the closer the samples are, the more similar microbial composition they have.

**Figure 4:** Abundant archaea genus at different draw solute reverse diffusion conditions. (Values are average of duplicates for sludge samples with fertilizer dosage; deviation of duplicates is within 2%).

**Figure 5:** Principle component analysis of bacterial community in sludge samples. Each point represents the microbial community in a specific sample. Distance between the sample dots signifies similarity; the closer the samples are, the more similar microbial composition they have.

**Figure 6:** Top 8 abundant bacteria genus at different draw solute reverse diffusion conditions (values are average of duplicates for sludge samples with fertilizer dosage; deviation of duplicates is within 2%).

**Table captions**

Table 1: Characteristics of anaerobic sludge used in this study.