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# Determination of the archaeal and bacterial communities in two-phase and single-stage anaerobic systems by 454 pyrosequencing

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ABSTRACT

2-Phase anaerobic digestion (AD), where the acidogenic phase was operated at 2 day hydraulic retention time (HRT) and the methanogenic phase at 10 days HRT, had been evaluated to determine if it could provide higher organic reduction and methane production than the conventional single-stage AD (also operated at 12 days HRT). 454 pyrosequencing was performed to determine and compare the microbial communities. The acidogenic reactor of the 2-phase system yielded a unique bacterial community of the lowest richness and diversity, while bacterial profiles of the methanogenic reactor closely followed the single-stage reactor. All reactors were predominated by hydrogenotrophic methanogens, mainly *Methanolinea*. Unusually, the acidogenic reactor contributed up to 24% of total methane production in the 2-phase system. This could be explained by the presence of *Methanosarcina* and *Methanobrevibacter*, and their activities could also help regulate reactor alkalinity during high loading conditions through carbon dioxide production. The enrichment of hydrolytic and acidogenic *Porphyromonadaceae*, *Prevotellaceae*, *Ruminococcaceae* and unclassified *Bacteroidetes* in the acidogenic reactor would have contributed to the improved sludge volatile solids degradation, and ultimately the overall 2-phase system's performance. Syntrophic acetogenic microorganisms were absent in the acidogenic reactor but present in the downstream methanogenic reactor, indicating the retention of various metabolic pathways also found in a single-stage system. The determination of key microorganisms further expands our understanding of the complex biological functions in AD process.



## Introduction

Anaerobic digestion (AD) has been widely applied for sludge treatment at many municipal and industrial wastewater treatment plants. The AD process involves biological hydrolysis, acidogenesis, acetogenesis and methanogenesis (Appels et al., 2008). Fundamentally, these reactions are performed by different microbial groups possessing various metabolic capabilities. Most chemoheterotrophic *Bacteria* are involved in the hydrolysis and acidogenesis reactions of proteins, carbohydrates and lipids (Nelson et al., 2011; Regueiro et al., 2012). Acetogenic microorganisms consist of acetate-producing syntrophic *Bacteria* or

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homoacetogenic *Bacteria* which carry out reversible reduction of carbon dioxide to acetate by utilising hydrogen. The cultivation of syntrophic microorganism requires the presence of its syntrophic partner (*e.g.*, hydrogenotrophic methanogen) to keep the hydrogen partial pressure low (Stams et al., 2012). Lastly, methanogenesis is performed by the methanogenic consortia of *Archaea* which generally utilised either acetate, formate, or hydrogen as electron donor (Liu and Whitman, 2008).

Accumulation of volatile fatty acids (VFAs), caused by an imbalance of the above four steps, is common in single-stage anaerobic systems operated at high organic loadings. VFA accumulation could reduce the reactor pH, inhibit methanogenic activity and subsequently cause system failure (Appels et al., 2008). The hydrolytic and acidogenic bacteria have growth rates which are magnitudes faster than methanogenic microorganisms (Zhang and Noike, 1991). 2-Phase AD configuration separates the two microbial groups through these different growth rates by manipulating solids retention time (SRT) in two connected but separate reactors. Operation of the acidogenic phase at lower SRTs (1–5 days) would maintain the optimal cultivation of hydrolytic/acidogenic bacteria, while in the next reactor, the methanogenic microorganisms and other slower-growing bacteria are cultivated at longer SRTs (>10 days) (Rubio-Loza and Noyola, 2010). Additionally, accumulation of organic acids in the acidogenic reactor aids release of non-crystalline organic polymers for faster sludge degradation. 2-Phase AD could therefore, be operated under higher organic loadings, while achieving better sludge degradation and biogas production than the single-stage AD (Bhattacharya et al., 1996; Rubio-Loza and Noyola, 2010). This had been demonstrated at pilot and full-scale (Ghosh et al., 1995). 2-Phase AD could also improve pathogen destruction (Rubio-Loza and Noyola, 2010) and alleviate foam problems during digester operation (Ghosh et al., 1995).

Despite the many reports on process performance, the underlying microbial community structure involved in phased AD for municipal sludge digestion has rarely been reported. For instance, there were cases when methanogenic activity was detected in the acidogenic reactor but this phenomena had not been adequately explained (Ghosh et al., 1995; Shimada et al., 2011). In-depth microbial consortium characterization studies have often focused on the single-stage AD configuration (Cardinali-Rezende et al., 2012; Shimada et al., 2011; Shin et al., 2010). The acidogenic reactor would, however, be operated in a manner quite dissimilar from the single-stage AD. There were a few studies investigating the methanogenic *Archaea* and bacterial populations in the 2-phase AD system, but the tools used had not yielded clear identification of key microbial populations. Zhang and Noike (1991) had used a cultivation-dependant method which might have biased towards excluding viable but non-culturable microorganism. The key microbial populations were not determined by Merlino et al. (2013), Shimada et al. (2011) and Schievano et al. (2012) due to limited number of (DNA) templates sequenced, in spite of denaturing gradient gel electrophoresis (DGGE) and clone library being employed. 454 pyrosequencing was proposed in this study to include more sequencing reads at faster analysis rate than cloning-based methods. A growing number of studies had recently adopted 454 pyrosequencing analysis on engineered environmental processes (Sundberg et al., 2013).

Previous study had compared the performance of single-stage against 2-phase AD systems for the treatment of sewage sludge (Maspolim et al., 2015). That study found that the volatile solids reduction and methane production were improved in 2-phase system operated at 2 + 10 day hydraulic retention time (HRT). The use of 454 pyrosequencing in this study attempted to resolve the microbial compositions within the two systems and to further understand the microbiological differences between the two systems. The characterization would focus on the determination of hydrolytic, acidogenic, acetogenic, and methanogenic communities in the single-stage and 2-phase reactors.

## 1. Materials and methods

### 1.1. Reactor start-up and operation

Two previously described (Maspolim et al., 2015) sets of anaerobic continuously stirred tank reactors (CSTRs) were operated as the single-stage and 2-phase systems, with HRTs of 12, 2 and 10 days for the single-stage, acidogenic and methanogenic reactors, respectively. These reactors were originally inoculated with anaerobic sludge from a local full-scale anaerobic digester treating municipal sludge. The feed was a mixture of primary and secondary sludge, collected from the same plant. During feeding, 36 mL of the sludge slurry was transferred from the feed reservoir into the acidogenic or single-stage reactor. Acidogenic reactor mixed liquor would be transferred into the methanogenic reactor as feed while mixed liquor from the single-stage and methanogenic reactors would be transferred into the effluent reservoir. These operations would be performed with peristaltic pumps every 14 min for 1 min. All reactors were operated at 35°C and pH of the acidogenic and methanogenic reactors was controlled at 5.5 ± 0.3 and 7.0 ± 0.2, respectively, by automatic dosing of 1 mol/L sodium hydroxide or hydrochloric acid. pH of the single-stage reactor could be maintained at pH 7.0 ± 0.2 without manipulation. The acidogenic reactor was maintained at pH 5.5 to optimize hydrolysis and acidogenesis reactions, as previously reported (Elefsiniotis and Oldham, 1994; Ghosh et al., 1995). The feed had 42,300 ± 3600 mg/L total COD; 2500 ± 1000 mg/L soluble COD; 32.1 ± 2.6 g/L TS; 25.7 ± 2.0 g/L VS; and pH 5.9 ± 0.2. Chemical oxygen demand (COD) and solids measurements were performed in accordance with Standard Methods (APHA, AWWA, WPCF, 2005). C2 to C7 volatile fatty acids (VFA) and the biogas volume and content were measured as previously described (Maspolim et al., 2015).

### 1.2. Nucleic acid extraction

Microbiological samples were obtained 82 days after the start of the anaerobic system operated with 12 days system HRT. It was assumed that the system would then hold representative microbial communities. DNA was extracted immediately after the samples were taken from the reactors. Prior to DNA