This document is downloaded from DR-NTU, Nanyang Technological University Library, Singapore.

Title	Comparison of single-stage and two-phase anaerobic sludge digestion systems : performance and microbial community dynamics(Main article)
Author(s)	Maspolim, Yogananda; Zhou, Yan; Guo, Chenghong; Xiao, Keke; Ng, Wun Jern
Citation	Maspolim, Y., Zhou, Y., Guo, C., Xiao, K., & Ng, W. J. (2014). Comparison of single-stage and two-phase anaerobic sludge digestion systems: Performance and microbial community dynamics. Chemosphere, 140, 54-62.
Date	2014
URL	http://hdl.handle.net/10220/24056
Rights	© 2014 Elsevier Ltd. This is the author created version of a work that has been peer reviewed and accepted for publication by Chemosphere, Elsevier Ltd. It incorporates referee's comments but changes resulting from the publishing process, such as copyediting, structural formatting, may not be reflected in this document. The published version is available at: [DOI:http://dx.doi.org/10.1016/j.chemosphere.2014.07.028].

*AbstractRevision, changes marked
Click here to view linked References

Abstract

This study compared reactor performance and the respective microbial community dynamics in the conventional single-stage and 2-phase anaerobic digestion (AD) systems, treating municipal sludge to generate methane. The 2-phase system's COD and VS reduction, and methane production could be maintained throughout the three HRTs tested ($p = 0.05$), which was associated with an increase in organic loading (30 d = 1.5 g COD L^{-1} d^{-1}, 20 d = 2.2 g COD L^{-1} d^{-1} and 10 d = 3.5 g COD L^{-1} d^{-1}); but this was not so in the single-stage system where it deteriorated at HRT of 10 d ($p = 0.05$) due to impairment of particulate COD reduction. qPCR, DGGE and the subsequent phylogenetic analysis revealed that microbial adaptation occurred as the seed sludge formed a different community in each reactor at 30 d HRT; however, no further significant microbial shift occurred at lower HRTs. The presence of specific hydrolytic and acidogenic *Flavobacteriales* and *Clostriales* in the acidogenic reactor may have allowed for enhanced hydrolysis and acidogenesis, leading to higher organic loading tolerance at 10 d HRT. Methanogenic activity in the acidogenic reactor may have been performed by *Methanobacteriales* and *Methanosarcinaceae*. Operation of the acidogenic reactor at neutral pH may have to be considered to ensure the cultivation of propionate oxidising bacteria, which could in turn, prevent reactor "souring" during high load conditions.

*Manuscript
Click here to view linked References

Comparison of Single-stage and Two-phase Anaerobic Sludge Digestion Systems - Performance and Microbial Community Dynamics

Yogananda Maspolim[a, b], Yan Zhou[a], Chenghong Guo[a, b*], Keke Xiao[a, b], Wun Jern Ng[a, b**]

[a] Advanced Environmental Biotechnology Centre, Nanyang Environment and Water Research Institute, Nanyang Technological University, 1 Cleantech Loop, Singapore 637141, Singapore

[b] Division of Environmental and Water Resources, School of Civil and Environmental Engineering, Nanyang Technological University, 50 Nanyang Avenue, Singapore 639798, Singapore

* Corresponding Author: Guo Chenghong (CHGUO@ntu.edu.sg)

**Corresponding Author: Ng Wun Jern (WJNg@ntu.edu.sg)

Corresponding Authors Tel: +65 94896165 (Guo Chenghong)
+65 67906813 (Ng Wun Jern)

Keywords

Two-phase anaerobic digestion; municipal sludge; biogas; microbial community; DGGE; qPCR

Abbreviations

AD	anaerobic digestion
CSTR	continuously stirred tank reactor
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
HRT	hydraulic retention time
OLR	organic loading rate
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
rRNA	ribosomal ribonucleic acid
SRT	solid retention time
TS	total solids
VFA	volatile fatty acids
VS	volatile solids
VSS	volatile suspended solids

1. Introduction

Municipal sludge is an inevitable by-product in current state-of-the-art of municipal wastewater treatment. Anaerobic digestion (AD) has commonly been applied to reduce the solids content and the pathogenic and vector attraction potential, while recovering renewable source of energy in the form of methane. AD involves a series of biological steps, namely hydrolysis, acidogenesis, acetogenesis and finally, methanogenesis (Angelidaki et al., 2000). Hydrolysis, often the rate-limiting step during sludge AD, would initially disintegrate and solubilise protein, carbohydrates and lipids into their simpler derivatives by physicochemical dissolution and microbial enzymatic reaction. The next two steps would then be performed by chemoorganotrophic microorganisms which obtained energy by fermentation or respiration reactions, utilising amino acids, saccharides, LCFAs, glycerol or different species of VFA as electron donor. Methanogenic bacteria then utilise acetic acid and hydrogen as the main electron donor for methane and carbon dioxide production (Angelidaki et al., 2000). Hence, stabilisation of organic solids and liquid components is achieved, yielding carbon dioxide and methane. However, high concentration of VFA may accumulate during sludge digestion in a single-stage AD configuration operated at high organic loading rate (OLR) or shortened hydraulic retention time (HRT). The accumulated VFA would decrease system pH, and ultimately cause process failure.

Optimisation of the AD process to achieve more efficient sludge stabilisation led to phased AD system (Ghosh et al., 1995). Acidogenic bacteria have growth rates which are magnitudes faster than the methanogenic bacteria. In the conventional high-rate AD reactor, an extended SRT is necessary to accommodate for the cultivation of slower growing methanogenic bacteria. However, reactor operation at extended SRTs

63 and neutral pH may suppress the acidogenic bacteria's capacity to produce VFA, the
64 intermediates for methanogenesis (Ghosh, 1987). Physical separation of the AD
65 process into its acidogenesis and methanogenesis phases can alleviate this problem,
66 and also help in controlling VFA conversion by the acetogenic and methanogenic
67 bacteria, avoiding "sour digestion". Process improvements in terms of solid and
68 pathogen reduction, biogas production and foaming alleviation, compared to the
69 conventional high-rate single-stage AD, had been reported previously (Ghosh et al.,
70 1995; Bhattacharya et al., 1996).

71 Besides solids reduction, process stability is another key parameter to be considered.
72 Fluctuating OLR and especially during sharp high excursions could affect process
73 performance. Methanogenic bacteria are slow-growing and at short SRTs may be
74 washed out from the reactor. The imbalance between acidogens and methanogens can
75 then lead to accumulation of VFA at inhibitory levels (Ghosh et al., 1995). The
76 opportunity to shorten SRTs while still maintaining digestion performance is
77 welcomed because it would allow reduction of reactor size for a given load.

78 Understanding the complex interactions of microorganisms involved in the AD
79 process is of interest for better process control. Previous studies had focused on
80 characterising the microbial population in the single-stage sludge AD process (Raskin
81 et al., 1995; Rivière et al., 2009; Shin et al., 2010b), but rarely in the phased AD
82 configuration (Zhang and Noike, 1991; Shin et al., 2010a), and especially for phased
83 AD treating sewage sludge (Shimada et al., 2011). Microbial characterisation of 2-
84 phase microbial community had been attempted using clone library technique at a
85 single organic loading (Shimada et al., 2011); but, varied organic loadings would
86 likely impact on the microbial dynamics.

87 Therefore, the purpose of this study is to evaluate the performance of two municipal
88 sludge digestion systems - single-stage and 2-phase AD configurations operated at
89 various organic loading conditions. The consequent microbial dynamics and systems'
90 performance would then be correlated.

91

2. Materials & Methods

2.1. Reactor start-up and operation

The single-stage reactor set was a single 50 L working volume digester vessel. The 2-phase setup had a 7.5 L acidogenic reactor (phase 1) preceding a 42.5 L methanogenic reactor (phase 2). The reactor operating conditions are as summarised in Table 1. The working volumes of the 2-phase reactors were adjusted as HRTs were reduced from 30 d to 20 d, and finally to 12 d. As the HRTs were reduced, the OLR had increased from 1.5 to 2.2 and 3.5 g COD L^{-1} d^{-1}, respectively. The reactors were operated in CSTR fashion, so the HRT = SRT. Temperature of all reactors was controlled at 35 °C; pH of the acidogenic reactor was pH 5.5, while the single-stage and methanogenic reactors were controlled at pH 7.0. pH was maintained by automated dosing of 1 M hydrochloric acid or 1 M sodium hydroxide. All reactors were seeded with anaerobic sludge collected from an anaerobic digester at a local wastewater reclamation plant. The sludge substrate used was a mixture of municipal primary and secondary sludge from the same water reclamation plant. Its characteristic was 42,300 ± 3,600 mg total COD L^{-1}; 2,500 ± 1,000 mg soluble COD L^{-1}; 32.1 ± 2.6 g TS L^{-1}; 25.7 ± 2.0 g VS L^{-1}; and pH 5.9 ± 0.2. Sludge substrate was collected weekly and stored at 4 °C before use.

2.2. Analytical methods

Samples were collected twice weekly from all reactors. Chemical oxygen demand (COD), total solids (TS), total suspended solids (TSS), volatile solids (VS), volatile suspended solids (VSS) were measured according to procedures in Standard Methods (APHA, 2005). Daily biogas volume produced was measured with a thermal-based gas flowmeter (McMillan, USA), fitted on the bioreactor. Biogas composition was

assessed using gas chromatography (GC) (7890A, Agilent, USA) with a thermal conductivity detector. Various volatile fatty acids (VFA) and their concentrations were measured using the same GC model (Agilent, USA) but fitted with the ZB-FFAP column (Phenomenex, USA) and a flame ionisation detector. VFA samples were prepared by centrifuging a sludge sample at 13,000 xg for 5min before filtering through a 0.2 μm nylon membrane.

2.3. DNA extraction and storage

Before extraction, the sludge sample was diluted to reduce TS concentration to below 2 g L^{-1} to ensure maximum extraction efficiency. 1 mL duplicates of the sample were then washed twice with phosphate buffered saline by centrifugation (20,000 xg for 2 min) and resuspension. Deoxyribonucleic acid (DNA) was extracted using an automated DNA extraction kit (MagNA Pure, Roche Diagnostic GmBH, Germany) following the manufacturer's recommended protocol. Extracted DNA was stored at -20 ^{0}C before PCR amplification was performed.

2.4. DGGE and phylogenetic identification

Bacterial and archaeal community structures were studied by targeting the 16s ribosomal ribonucleic acid (rRNA) gene. Touch-down PCR protocol and the bacterial- (GC-BAC338F, BAC805R), archaeal-specific (GC-ARC787F, ARC1059R) primers and their respective GC clamps followed those used by Shin et al. (2010a). PCR product was run on DGGE using DCode system (Bio-Rad, USA) with 8% (w/v) acrylamide, where the 100% denaturant contained 7 M urea and 40% (v/v) formamide. Bacterial DGGE was run on 30-60% denaturing gradient, while archaeal DGGE on 40-70% gradient. The gel was run at 85 V for 14 h in TAE buffer (1X). The

gel was subsequently stained with ethidium bromide and documented using GelDoc™ XR+ system (BioRad, USA).

Bands of interest were cut and eluted in 50 µL of nuclease-free water overnight. 2 µL of eluted DNA solution was amplified using the same bacterial and archaeal primers as above, without the GC clamps (Shin et al., 2010a). PCR products were purified from 2% agarose gel, cloned into pGEM-T Easy vector (Promega, USA) and sequenced. Resulting sequences were compared against reference database in the GenBank, using BLAST (http:// http://blast.ncbi.nlm.nih.gov/). Neighbour-joining trees were then constructed with MEGA5 software using Jukes-Cantor algorithm and bootstrapped 1,000 times.

2.5. qPCR

Quantitative polymerase chain reaction (qPCR) was performed using primer/probe sets targeting the same 16s rRNA genes, specific for *Bacteria* and *Archaea* as mentioned above, but without the GC clamps. The two-step PCR amplification protocol, the primers and TaqMan probes of 16s rRNA genes for identifying archaeal orders *Methanobacteriales*, *Methanomicrobiales*; and families *Methanosarcinaceae*, *Methanoaetaceae* followed those described by Yu et al. (2005). qPCR was performed using LightCycler 480 (Roche Diagnostics GmBH, Germany) with LightCycler480 TaqMan Probe Master (Roche Diagnostics GmBH, Germany) system. Each extracted DNA duplicate was analysed by qPCR. Standard curves for the qPCR analysis were prepared as previously described (Yu et al., 2005). The calibrated number of DNA per gram VSS would give an estimated measure of quantitative abundance.

3. Results

3.1. The performance of single stage and 2-phase sludge AD systems

The two systems were operated for 402 d at 3 different OLR. Table 1 summarises the operating parameters and performances. Student's t-test was used to evaluate the COD, VS reduction and methane production data between the single-stage and 2-phase systems and these were only significantly different at 12 d HRT ($p < 0.05$, $n = 16$). Mean COD and VS reduction in the single-stage AD system during 30 and 20 d HRT was around 39% and 32%, before it decreased to 30.8% and 26.3% at 12 d HRT, respectively. On the other hand, mean COD and VS reduction in the 2-phase system could be maintained at between 40.7% to 42.3% and 31.6% to 35.5%, respectively, even when the HRT was decreased from 30 to 12 d. The mean methane production gradually increased due to increasing OLR, ranging from 9.4 to 11.7 and 15.6 L d^{-1} at 30, 20 and 12 d HRT in the single-stage reactor. On the contrary, mean methane production in the 2-phase system was similar to single-stage AD at 30 and 20 d HRT (10.0 and 12.7 L d^{-1}, respectively); but was higher at 12 d HRT (20.6 L d^{-1}).

***(insert table 1 here)**

The acidogenic reactor contributed 20% to 24% of all the methane generated in the 2-phase system (Table 1). Despite methanogenesis being commonly known to proceed between pH 6.6 to 7.8, previous researchers have also reported methanogenic activity in the acidogenic reactor at pH 5.8 (Ghosh et al., 1995). The biogas composition in the single-stage and methanogenic reactors was 64% - 67% methane, 30% - 34% carbon dioxide, while the acidogenic reactor was 48% - 49% methane and 48% - 50% carbon dioxide. There was low hydrogen partial pressure found in all the anaerobic

185 reactors (< 0.01%). This demonstrated that there were active methanogens in the
186 acidogenic reactor.

187 ***(insert table 2 here)**

188 Table 2 presents the distribution of VFA in the sludge substrate and acidogenic
189 reactor. The most abundant VFA species in the acidogenic reactor was propionic acid,
190 followed by isovaleric, butyric and acetic acid. The acetic acid concentration in the
191 acidogenic reactor was lower than it was at in the sludge substrate as acetic acid was
192 converted into methane. Table 2 also shows that total VFA in the acidogenic reactor
193 decreased as the HRT was reduced. However, calculating the sum of the COD
194 reduced and residual VFA in the acidogenic reactor resulted in values of 27.5, 28.4
195 and 36.6 g COD for the 5, 3 and 2 d HRTs, respectively. These values represented the
196 amount of substrate COD converted into residual VFA and biogas in the acidogenic
197 reactor. The total amount of substrate COD converted into VFA and biogas were at
198 least maintained as HRT was decreased, and showed that acidogenesis was not
199 adversely affected upon the reduction of HRT.

200 *3.2. Bacterial and archaeal profiles*

201 DGGE and subsequent phylogenetic identification were performed to reveal the
202 microbial diversity and community dynamics in the single-stage and 2-phase AD
203 systems. Microbial characterisations of *Archaea* (Fig. 1A) and *Bacteria* (Fig. 1B)
204 were conducted when the anaerobic processes were stable and had undergone at least
205 4 HRT cycles. A total of 30 bands from both the bacterial and archaeal DGGE gels
206 were sequenced and the nearest taxonomic identification from the database were
207 identified (Table 3). Phylogenetic trees were also constructed for *Archaea* (Fig. 2) and
208 *Bacteria* (Fig. 3).

***(insert figure 1 here)**

Fig. 1A shows that the archaeal population of different reactors were similar in all samples tested, irrespective of the HRT. Two methanogenic orders were identified by the phylogenetic analysis, namely *Methanomicrobiales* and *Methanosarcinales* orders (Demirel and Scherer, 2008). The bands A1 to A11 appeared in the sludge substrate, seed sludge and the 3 anaerobic reactors on all HRTs tested. These bands sequences were found to be closely related to *Methanospirillum hungatei*, *Methanolinea mesophila* and *Methanoregula formicicum*. These species were of *Methanomicrobiales* order and utilised hydrogen or formate as electron acceptor to produce methane (Demirel and Scherer, 2008). A13 and A14 appeared in the single-stage and methanogenic reactors when the system HRT was reduced to 20 d and 12 d. A13 and A14 were closely related to *Methanoculleus receptaculi*, another species of *Methanomicrobiales* order. A15 was affiliated to *Methanosarcina barkei* which seemed to decrease in intensity over decreasing HRTs in the methanogenic reactor. *Methanosarcina* tend to be more flexible and can utilise a wider range of substrates as electron donor (Demirel and Scherer, 2008).

***(insert table 3 here)**

***(insert figure 2 here)**

The bacterial DGGE gel in Fig. 1B shows that the bacterial profiles of sludge substrate, seed sludge and the various anaerobic reactors were distinct. Acidogenic reactor population did not seem to change significantly as HRT decreased. There were also bands which consistently appeared in all the samples as well as those which were distinct for a particular sample. For instance, B3 to B6 appeared in all samples, while B9, B12 and B15 were observed only in the acidogenic reactor. Phylogenetic identification demonstrated that *Clostridiales*, *Flavobacteriales, Pseudomonadales*

and *Syntrophobacteriales* were the four bacterial orders identified in the anaerobic reactors. Unfortunately, many of the bacterial sequences could not be affiliated to cultured, classified microorganism. B3 was closely related (97%) to *Enhydrobacter* species of *Pseudomonadales* order. B4 and B6 matched (99%) to uncultured *Bacteroidetes* bacterium in sludge AD (Table 3) and Uncultured *Fluviicola* sp. from membrane bioreactor biofilm which were of *Flavobacteriales*. B5 matched (98%) to uncultured *Ruminococcaceae* bacterium identified in wetlands of the *Clostridiales* (Table 3).

Of those bands only found in the acidogenic reactor, B9, B12 were closely related to uncultured bacterium identified in AD process treating coking wastewater and full-scale sludge AD process, respectively (Table 3). These sequences seemed to cluster within *Flavobacteriales* class. B15 had 97% maximum identity to Uncultured *Clostridiales* bacterium identified in AD treating swine manure (Table 3). Additionally, B1 and B2 were present in the acidogenic reactor as well as the sludge substrate. Both sequences matched (100%) for *Cloacibacterium normanense*, a facultative anaerobic bacterium of *Flavobacteriales* order. This organism was isolated from the influent municipal wastewater, capable of hydrolysing starch and gelatin (Allen et al., 2006).

There were also bands which were found to be present only in the single-stage and methanogenic reactors. B7 was closely related (96%) to *Smithella propionica*, a known syntrophic oxidiser, which cooperated with hydrogenotrophic microorganisms, such as hydrogenotrophic methanogen to convert propionic acid to acetic acid (Liu et al., 1999). *Clostridiales* and *Flavobacteriales* orders could also be found in these anaerobic reactors. B10 and B13 were affiliated to *Saccharofermentans acetigenes* (98%) and uncultured *Firmicutes* bacterium in full-scale AD treating sludge (99%),

respectively, which were both clustered within the *Clostridiales*. *Saccharofermentans acetigenes* was isolated from an anaerobic reactor, capable of fermenting saccharides (Chen et al., 2010). Lastly, B14 was closely related (99%) to uncultured *Bacteroidetes* in full-scale AD treating sludge, which came under the *Flavobacteriales* order.

***(insert figure 3 here)**

3.3. Microbial quantitative analysis by qPCR

The quantitative dynamics over different operational HRTs of the bacterial and methanogenic population was assessed by qPCR. The qPCR analysis targeted universal *Bacteria, Archaea* and 4 methanogenic orders and families, commonly found in the sludge AD process (Shin et al., 2010b). Fig. 4 shows the average quantification of microbial population during each HRT when the process was stable and had undergone at least 4 HRT cycles. The mean bacterial 16s rRNA genes of the sludge substrate reduced from 1 x 10^{10} to c. 5 x 10^{9} DNA copies mg VSS^{-1} in the single-stage and methanogenic reactors regardless of HRT, indicating cell lysis as microbial cells decayed during the AD process. Quantitative shift was observed when the biomass was adapted from the seed sludge into single-stage, acidogenic and methanogenic reactor biomass (Fig. 4). However, there was no obvious quantitative shift of the bacterial and methanogenic population tested as the HRT was decreased. Total methanogenic population in the seed sludge (3.6 x 10^{8} DNA copies mg VSS^{-1}) was lower than the single-stage and methanogenic reactors (c. 6 x 10^{8} DNA copies mg VSS^{-1}), but was slightly higher than the sludge substrate and acidogenic reactor (1 x 10^{8} DNA copies mg VSS^{-1}) (Fig. 4A).

***(insert figure 4 here)**

282 Hydrogenotrophic *Methanomicrobiales* dominated in all samples, between 80 to 90%
283 in abundance to all the methanogens, but at varying concentrations (Fig. 4B). The
284 results also demonstrated that the mean 16s rRNA gene concentration of
285 *Methanomicrobiales* (Fig. 4B) and *Methanosaetaceae* (Fig. 4C) declined as the seed
286 sludge (3×10^8 and 9×10^6 DNA mg VSS^{-1}, respectively) evolved to the acidogenic
287 reactor biomass (ca. 1×10^8 and 1×10^6 DNA mg VSS^{-1}, respectively). Meanwhile,
288 the mean 16s rRNA gene concentration of *Methanobacteriales* (Fig. 4B) and
289 *Methanosarcinaceae* (Fig. 4C) increased as the seed sludge (3×10^6 and 2×10^5 DNA
290 mg VSS^{-1}, respectively) formed the acidogenic reactor biomass (c. 1×10^7 and 1×10^6
291 DNA mg VSS^{-1}, respectively). *Methanobacteriales*, *Methanomicrobiales* (Fig. 4B)
292 and *Methanosaetaceae* (Fig. 4C) were in the similar range for both the single-stage
293 and the methanogenic reactors (3×10^7, 5×10^8 and 1×10^7 DNA copies mg VSS^{-1},
294 respectively). Only the mean 16s rRNA gene concentration of *Methanosarcinaceae*
295 (Fig. 4C) was found to be one magnitude higher in the methanogenic reactor (3×10^7
296 DNA copies mg VSS^{-1}) than any other samples.

297

298 **4. Discussion**

299 This study compared application of the single-stage and 2-phase AD configurations in
300 treatment of municipal sludge. The same working conditions in terms of the seed
301 sludge source, substrate and temperature were applied to both configurations. The
302 effect of decreasing HRT which inevitably increased the OLR was evaluated. The
303 competitive advantage of using the 2-phase configuration at the shortened HRT of 12
304 d was shown to maintain sludge digestion and biogas production.

305 Stable COD, VS reduction and methane yield could be maintained at the OLR from
306 1.5 to 3.5 g COD L^{-1} d^{-1} in the 2-phase AD configuration (Table 1). On the contrary,
307 the single-stage AD configuration could not maintain the same performance once the
308 OLR increased from 2.2 to 3.5 g COD L^{-1} d^{-1}. To determine if methanogenesis was
309 the limiting factor in the single-stage reactor AD, mean residual VFA of both single-
310 stage and methanogenic reactors in the mixed liquor were compared, but both were
311 found to be low (< 100 mg COD L^{-1}), irrespective of HRT. VFA was directly
312 consumed to produce biogas here and implied that methanogenesis was not restricted
313 in the single-stage reactor (Table 1). However, higher mean particulate COD
314 concentration was found in the single-stage (28,500 mg COD L^{-1}) than the
315 methanogenic reactor (24,000 mg COD L^{-1}) at 12 d HRT (Table 1). Student's t-test
316 confirmed that the difference between these two values was statistically significant (p
317 < 0.05, $n = 16$). Hydrolysis of particulate organics, which require longer reaction
318 (Mahmoud et al., 2004), seemed to be retarded in the single-stage reactor at 12 d
319 HRT. These results showed that the 2-phase AD system could tolerate shorter HRTs
320 and higher organic loadings than the single-stage AD configuration. This agreed with
321 a previous AD system which treated other starch-rich or proteinaceous substrates
322 (Zhang and Noike, 1991; Bhattacharya et al., 1996; Lv et al., 2010).

323 This improved performance in the 2-phase system could be due to chemically and
324 biologically induced reasons. By maintaining the acidogenic reactor at pH 5.5, protein
325 may lose its conformational structure and be denatured as the protein was protonated
326 (Neyens et al., 2004); or break glycosidic linkages of carbohydrates and deform their
327 tertiary structure. All this, and disruption to the extracellular matrix, would leave the
328 biodegradable polymers exposed for extracellular enzymes to hydrolyse (Sheng et al.,
329 2010). Some biological cells themselves lose their viability and turgor pressure under
330 acidic condition, hence assisting the lysis of microbial cells (Neyens et al., 2004).
331 Secondly, the physical separation of phases in the 2-phase system may enhance the
332 activity of hydrolytic/acidogenic bacteria as their growth condition was optimised
333 under the shortened HRT and pH 5.5 (Ghosh, 1987). Acidogenic bacteria were known
334 to have maximum specific growth rate (μ_{max}) of 5.1 d^{-1}, while methanogenic bacteria
335 was 0.6 d^{-1} (Angelidaki et al., 2000). Cultivating the acidogenic bacteria under
336 extended SRTs to accommodate the methanogenic bacteria in a single-stage
337 configuration is disadvantageous, as acidogenic bacteria would then lose their optimal
338 microbial activity under this stationary growth phase. This study hence also focused
339 on the use of molecular techniques to evaluate the microbial community significance
340 between the phase-separated and single-stage AD process.

341 Microbial community profiling by DGGE revealed that the seed sludge, sludge
342 substrate, single-stage and 2-phase AD reactors showed distinct bacterial fingerprints
343 (Fig. 1B), but similar archaeal fingerprints (Fig. 1A). First of all, a core bacterial
344 group was observed in all the anaerobic reactors (B3 – B6), which were
345 *Flavobacteriales*, *Clostridiales* and *Enhydrobacter*-related *Bacteria* (Table 3) and
346 appeared to contribute to acidogenic activity (Sneath et al., 1986; Kirchman, 2002;
347 Lynd et al., 2002). Other acidogenic *Flavobacteriales* and *Clostridiales* species (B10,

348 B13 and B14) were also identified in the single-stage and methanogenic reactors. The
349 phyla *Bacteroidetes*, *Proteobacteria* and *Firmicutes* which contained
350 *Flavobacteriales*, *Pseudomonadales* and *Clostridiales* orders, respectively, formed the
351 most abundant taxonomy identified in the full-scale AD process (Rivière et al., 2009),
352 and the findings in this study (Table 3) were similar. The accumulation of propionic
353 acid in the acidogenic reactor could be explained by the absence of B7, a sequence
354 closely matching the syntrophic propionate oxidiser *Smithella propionica*. The growth
355 condition in the acidogenic reactor was unfavourable for *Smithella propionica*, as it
356 was not able to grow below pH 6.3 (Liu et al., 1999). Nevertheless, the overall 2-
357 phase AD system was not affected as *Smithella propionica* was detected in the
358 methanogenic reactor, consuming propionic acid to < 0.1 mM. Excessive propionic
359 acid in the acidogenic reactor can be detrimental as reactor pH could then drop and
360 the level of undissociated acids rise, inhibiting the methanogenic population in the
361 acidogenic reactor, and subsequently also affecting the methanogenic reactor
362 (Angelidaki et al., 2000). Further investigation where the acidogenic reactor would be
363 operated at around neutral pH to ensure the cultivation of the propionate oxidisers is
364 desirable to determine if this would impact on the improved sludge digestion
365 performance, as obtained during the operation of the 2-phase against the single-stage
366 AD system.

367 The microbial differentiation between the reactors was more obvious with the
368 acidogenic reactor, where B9, B12 and B15 of *Flavobacteriales* and *Clostridiales*
369 were detected (Fig. 1B). Even though they could not be affiliated to known species,
370 members of these orders were known to be chemoorganotrophic heterotrophs, and
371 would most likely be involved in the degradation and fermentation of organic matters
372 (Kirchman, 2002; Lynd et al., 2002). The uncultured *Flavobacteriales* of B9 seemed

373 to play a more important role as suggested by its thick band intensity in all HRTs
374 tested in the acidogenic reactor. Since B9, B12 and B15 only appeared in the
375 acidogenic reactor, these acidogenic bacteria were presumably suppressed in a single-
376 stage AD system, but could maximise their acidogenic potential upon cultivation in
377 the acidogenic reactor at 2 to 5 d HRT, with pH 5.5. Their presence may have been
378 the reason for the better overall digestion performance of the 2-phase AD system.

379 A methanogenic bacterial population shift could be observed as the seed sludge
380 adapted to single-stage, acidogenic and methanogenic operation at 30 d HRT (Fig. 4).
381 Acetic acid is widely regarded to be the most important VFA species in the AD
382 process (Angelidaki et al., 2000), but was not the major VFA in this acidogenic
383 reactor (Table 2). Acetate could be converted into hydrogen and carbon dioxide by
384 syntrophic acetate oxidising bacteria, before methanogenesis by hydrogenotrophic
385 methanogenesis (Angelidaki et al., 2000); but no such microbial group was detected
386 by DGGE and the subsequent phylogenetic identification. The most abundant
387 methanogenic order in all the anaerobic reactors, including in the sludge substrate,
388 was the hydrogenotrophic *Methanomicrobiales* (Fig. 4), suggesting the importance of
389 hydrogenotrophic methanogenesis as the main metabolic pathway in this AD system.
390 Other studies had previously revealed *Methanomicrobiales* to be the most abundant
391 methanogenic group in a sludge AD process by dot-blot hybridisation or qPCR
392 (Raskin et al., 1995; Shin et al., 2010b). DGGE analysis also supported this
393 observation as most of the high-intensity archaeal bands present in all samples were
394 identified as of *Methanomicrobiales* order (Table 3), closely related to
395 *Methanospirillum hungatei* and *Methanolinea mesophila*.

396 Amongst the aceticlastic methanogen in the acidogenic reactor, acetate was
397 presumably converted into methane by the acetaticlastic *Methanosarcinaceae* family.

398 *Methanosarcinaceae* increased by almost one log difference from the start to when
399 the acidogenic reactor had stabilised, while another aceticlastic methanogenic family,
400 *Methanosaetaceae* decreased by close to one log difference (Fig. 4C). *Methanosaeta*
401 sp. grow in the pH range of 6.5 and 8.5, while *Methanosarcina* sp. does so between
402 pH 5 and 8; and could tolerate higher acetate concentration (up to 250 mM acetate)
403 than *Methanosaeta* (De Vrieze et al., 2012). DGGE analysis correspondingly found a
404 band sequence which was closely related to *Methanosarcina barkeri*, while no band
405 was found for *Methanosaetacaea*. The band appeared weakly in the acidogenic
406 reactor but more strongly in the methanogenic reactor samples (Fig. 1A), as
407 confirmed by the qPCR data (Fig. 4C). *Methanosarcina barkeri* had been reported to
408 utilise a wide range of substrates other than acetate, such as hydrogen and carbon
409 dioxide, methanol and methylamines (Demirel and Scherer, 2008). It is possible that
410 the acidic pH and shortened HRT in the acidogenic reactor generated such compounds
411 as by-product, which was then transferred to the methanogenic reactor and stimulated
412 the proliferation of *Methanosarcina barkeri*. Another methanogen which was able to
413 grow in the acidogenic reactor was the hydrogenotrophic *Methanobacteriales*
414 (Demirel and Scherer, 2008), rising by almost one log difference from the start to
415 when the acidogenic reactor had stabilised (Fig. 4). These results seemed to indicate
416 the contribution of *Methanobacteriales* and *Methanosarcinaceae* for the
417 methanogenesis step in the acidogenic reactor. Unfortunately, no *Methanobacteriales*
418 species was indicated by the DGGE analysis. The presence and activity of
419 methanogenic bacteria in the acidogenic reactor and the presence of acidogenic
420 bacteria in the methanogenic reactor showed that complete phase separation
421 (acidogenesis and methanogenesis) could not be achieved in a 2-phase AD process
422 treating municipal sludge.

423 The effect of decreasing the operational HRT on AD could be drastic and reduce the
424 sludge digestion performance. However, shortening the HRT is advantageous in
425 sizing the reactor volume, especially in places with limited land space or operational
426 capacity of existing anaerobic reactors. Fluctuations in the influent substrate volume
427 could also affect process HRT. Too short HRT would wash out slow-growing
428 microorganisms with growth rates shorter than the operational HRT applied. For
429 instance, B9 appeared to decrease in intensity as the HRT reduced from 3 to 2 d in the
430 acidogenic reactor, suggesting that this species may have been washed out at the
431 shorter HRT of 2 d (Fig. 1B). Fortunately, the reduction of this bacterial species did
432 not seem to affect the overall 2-phase system digestion performance. Secondly, the
433 increase in OLR associated with the HRT reduction in the system in this report could
434 also create an unfavourable condition for the sensitive methanogenic bacteria, as more
435 potentially inhibitory compounds would be present in the reactor. Such situation is
436 normally associated with the accumulation of VFA in the anaerobic reactor whereby
437 the conversion of acetic acid into methane is inhibited. However, the VFA
438 concentrations in the single-stage and the methanogenic reactor were almost
439 negligible, which indicated both systems' methanogenic populations could tolerate the
440 increase in OLR. Evaluation of the methanogenic bacteria quantity by qPCR showed
441 that each methanogenic group was not affected by the decreasing HRT. The presence
442 of methanogenic bacteria in the sludge substrate may have helped in maintaining the
443 reactors' methanogenic population, and hence, methanogenesis reaction could be
444 preserved (Fig. 1A and 4A). Thirdly, reducing the HRT could also impair the
445 degradation of particulate compounds which require extended reaction time to
446 complete. As previously discussed, this was the case with the single-stage process at
447 12 d HRT. On the other hand, the 2-phase process performance could still be

maintained due to the specific chemoorganotrophic bacterial population (B9, B12 and B15) cultivated in the acidogenic reactor. There was no significant change in the bacterial and archaeal population in the acidogenic reactor as the HRT was reduced (Fig. 1 and 4). This implied that the acidogenic reactor selected for a specialised community structure which could tolerate the reduction in HRT from 5 to 2 d.

5. Conclusion

The 2-phase AD system maintained COD and VS reduction, and methane production when operated at 30, 20 and 10 d HRT; while the single-stage system performance deteriorated as the HRT was reduced from 20 to 10 d. The latter was due to the decrease in particulate COD reduction efficiency. Microbial community analysis revealed distinct microbial profiles in the single-stage, acidogenic and methanogenic reactors, which generally persisted as the HRT was decreased. The identification of hydrolytic and acidogenic *Flavobacteriales* and *Clostridiales* only in the acidogenic reactor could possibly explain the enhanced sludge digestion performance of the 2-phase over the single-stage system at 10 d HRT. This study also discovered the inability of propionate oxidising bacteria to grow in the acidogenic reactor, and hence, suggested reconsideration for the latter to be operated at neutral pH.

Acknowledgement

We are grateful for funding support from the National Research Foundation, Singapore for the project "Wastewater Treatment Plants as Urban Eco Power Stations". The authors also acknowledge Dr Jaai Kim for her standard plasmids used in the qPCR analysis.

References

Allen, T.D., Lawson, P.A., Collins, M.D., Falsen, E., Tanner, R.S., 2006. Cloacibacterium normanense gen. nov., sp. nov., a novel bacterium in the family Flavobacteriaceae isolated from municipal wastewater. International journal of systematic and evolutionary microbiology 56, 1311-1316.

Angelidaki, I., Ellegaard, L., Ahring, B.K., 2000. A comprehensive model of anaerobic bioconversion of complex substrates to biogas. Biotechnology and Bioengineering 63, 363-372.

APHA, 2005. WPCF (2005) Standard methods for the examination of water and wastewater. Public Health Association, Washington, DC.

Bhattacharya, S.K., Madura, R.L., Walling, D.A., Farrell, J.B., 1996. Volatile solids reduction in two-phase and conventional anaerobic sludge digestion. Water Res. 30, 1041-1048.

Chen, S., Niu, L., Zhang, Y., 2010. Saccharofermentans acetigenes gen. nov., sp. nov., an anaerobic bacterium isolated from sludge treating brewery wastewater. International journal of systematic and evolutionary microbiology 60, 2735-2738.

De Vrieze, J., Hennebel, T., Boon, N., Verstraete, W., 2012. *Methanosarcina*: The rediscovered methanogen for heavy duty biomethanation. Bioresource Technology 112, 1-9.

Demirel, B., Scherer, P., 2008. The roles of acetotrophic and hydrogenotrophic methanogens during anaerobic conversion of biomass to methane: a review. Reviews in Environmental Science and Biotechnology 7, 173-190.

Ghosh, S., 1987. Improved sludge gasification by two-phase anaerobic digestion. Journal of environmental engineering 113, 1265-1284.

Ghosh, S., Buoy, K., Dressel, L., Miller, T., Wilcox, G., Loos, D., 1995. Pilot-and full-scale two-phase anaerobic digestion of municipal sludge. Water Environment Research 67, 206-214.

Kirchman, D.L., 2002. The ecology of Cytophaga–Flavobacteria in aquatic environments. FEMS microbiology ecology 39, 91-100.

Liu, Y., Balkwill, D.L., Aldrich, H.C., Drake, G.R., Boone, D.R., 1999. Characterization of the anaerobic propionate-degrading syntrophs Smithella propionica gen. nov., sp. nov. and Syntrophobacter wolinii. International Journal of Systematic Bacteriology 49, 545-556.

Lv, W., Schanbacher, F.L., Yu, Z., 2010. Putting microbes to work in sequence: recent advances in temperature-phased anaerobic digestion processes. Bioresource Technology 101, 9409-9414.

Lynd, L.R., Weimer, P.J., van Zyl, W.H., Pretorius, I.S., 2002. Microbial Cellulose Utilization: Fundamentals and Biotechnology. Microbiology and Molecular Biology Reviews 66, 506-577.

Mahmoud, N., Zeeman, G., Gijzen, H., Lettinga, G., 2004. Anaerobic stabilisation and conversion of biopolymers in primary sludge—effect of temperature and sludge retention time. Water Res. 38, 983-991.

Neyens, E., Baeyens, J., Dewil, R., De heyder, B., 2004. Advanced sludge treatment affects extracellular polymeric substances to improve activated sludge dewatering. Journal of Hazardous Materials 106, 83-92.

Raskin, L., Zheng, D., Griffin, M.E., Stroot, P.G., Misra, P., 1995. Characterization of microbial communities in anaerobic bioreactors using molecular probes. Antonie van Leeuwenhoek 68, 297-308.

Rivière, D., Desvignes, V., Pelletier, E., Chaussonnerie, S., Guermazi, S., Weissenbach, J., Li, T., Camacho, P., Sghir, A., 2009. Towards the definition of a core of microorganisms involved in anaerobic digestion of sludge. The ISME Journal 3, 700-714.

Sheng, G.-P., Yu, H.-Q., Li, X.-Y., 2010. Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: A review. Biotechnology Advances 28, 882-894.

521 Shimada, T., Morgenroth, E., Tandukar, M., Pavlostathis, S., Smith, A., Raskin, L., Kilian, R.,
522 2011. Syntrophic acetate oxidation in two-phase(acid--methane) anaerobic digesters. Water
523 Science & Technology 64, 1812-1820.
524 Shin, S.G., Han, G., Lim, J., Lee, C., Hwang, S., 2010a. A comprehensive microbial insight into
525 two-stage anaerobic digestion of food waste-recycling wastewater. Water Res. 44, 4838-
526 4849.
527 Shin, S.G., Lee, S., Lee, C., Hwang, K., Hwang, S., 2010b. Qualitative and quantitative
528 assessment of microbial community in batch anaerobic digestion of secondary sludge.
529 Bioresource Technology 101, 9461-9470.
530 Sneath, P.H., Mair, N.S., Sharpe, M.E., Holt, J.G., 1986. Bergey's manual of systematic
531 bacteriology. Volume 2. Williams & Wilkins.
532 Yu, Y., Lee, C., Kim, J., Hwang, S., 2005. Group-specific primer and probe sets to detect
533 methanogenic communities using quantitative real-time polymerase chain reaction.
534 Biotechnology and Bioengineering 89, 670-679.
535 Zhang, T.C., Noike, T., 1991. Comparison of one-phase and two-phase anaerobic digestion
536 processes in characteristics of substrate degradation and bacterial population levels. Water
537 Science & Technology 23, 1157-1166.

538

539

Table
Click here to download Table: CHEM31621_R1 tables_R1.docx

List of Tables

Table 1. Summary of reactor operating parameters and performance in single-stage and 2-phase AD.

		Single-stage			2-phase system		
HRT (d)		30	20	12	30	20	12
pH			7.0		-		
OLR COD (g COD L^{-1} d^{-1})		1.5 ± 0.2	2.2 ± 0.2	3.5 ± 0.3	1.5 ± 0.2	2.1 ± 0.3	3.5 ± 0.3
OLR VS (g VS L^{-1} d^{-1})		0.9 ± 0.1	1.3 ± 0.1	2.1 ± 0.2	0.9 ± 0.1	1.3 ± 0.1	2.1 ± 0.2
Soluble COD (mg COD L^{-1})		490 ± 50	620 ± 100	460 ± 70			
Particulate COD (mg COD L^{-1})		27,300 ± 1,500	25,600 ± 2,200	28,500 ± 1,500			
Total COD reduction (%)		38.6 ± 5.1	39.2 ± 6.5	30.8 ±6.1	42.3 ± 5.6	40.9 ± 4.7	40.7 ± 5.7
VS reduction (%)		31.7 ± 6.4	32.2 ± 5.5	26.3 ± 6.1	31.8 ± 6.9	31.6 ± 4.8	35.5 ± 6.6
Total VFA (mg COD L^{-1})		26 ± 18	20 ± 12	18 ± 10			
Methane production (L d^{-1})		9.4 ± 1.2	11.7 ± 1.5	15.6 ± 2.0	10.0 ± 1.5	12.7 ± 0.9	20.6 ± 1.9
Methane yield (L g COD $added^{-1}$)		0.13 ± 0.04	0.12 ± 0.01	0.10 ± 0.01	0.16 ± 0.02	0.14 ± 0.02	0.14 ± 0.03
Methane yield (L g VS $added^{-1}$)		0.22 ± 0.08	0.2 ± 0.01	0.16 ± 0.01	0.27 ± 0.04	0.22 ± 0.01	0.22 ± 0.04
Biogas composition	**Methane (%)**	66 ± 1	64 ± 1	65 ± 1			
	Carbondioxide (%)	32 ± 1	34 ± 1	34 ± 1			
	Nitrogen (%)	1 ± 1	2 ± 1	1 ± 1			
		Acidogenic 2-phase			**Methanogenic 2-phase**		
HRT (d)		5	3	2	25	17	10
pH			5.5			7.0	
OLR COD (g COD L^{-1} d^{-1})		9.2 ± 1.0	14.3 ± 1.6	20.9 ± 2.0	1.7 ± 0.2	2.2 ± 0.1	3.5 ± 0.2
OLR VS (g VS L^{-1} d^{-1})		5.3 ± 0.6	8.7 ± 0.7	12.8 ± 1.2	1.0 ± 0.08	1.3 ± 0.08	2.2 ± 0.2
Soluble COD (mg COD L^{-1})		4,800 ± 1,000	4,400 ± 700	2,900 ± 510	440 ± 50	580 ± 110	450 ± 70
Particulate COD (mg COD L^{-1})		37,600 ± 3,500	33,400 ± 2,000	32,200 ± 2,200	25,800 ± 1,100	24,600 ± 960	24,000 ± 1,400
Total COD reduction (%)		7.5 ± 6.8	11.4 ± 6.0	15.4 ± 6.0	37.6 ± 5.4	33.3 ± 2.8	29.9 ± 4.7
VS reduction (%)		9.4 ± 6.7	12.9 ± 5.0	12.5 ± 3.7	24.7 ± 5.2	21.5 ± 4.0	26.3 ± 6.6
Total VFA (mg COD L^{-1})		2,706 ± 546	2,471 ± 258	1,667 ± 410	32 ± 24	44 ± 17	94 ± 62
Methane production (L d^{-1})		2.4 ± 0.4	3.1 ± 0.6	4.1 ± 0.4	7.8 ± 1.4	9.6 ± 0.8	16.5 ± 2.1
Methane yield (L g COD $added^{-1}$)		0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.002	0.13 ± 0.02	0.11 ± 0.01	0.13 ± 0.02
Methane yield (L g VS $added^{-1}$)		0.06 ± 0.02	0.05 ± 0.01	0.04 ± 0.002	0.22 ± 0.03	0.19 ± 0.02	0.20 ± 0.03
Biogas composition	**Methane (%)**	48 ± 2	48 ± 2	49 ± 2	67 ± 1	67 ± 1	66 ± 3
	Carbondioxide (%)	48 ± 3	50 ± 1	48 ± 2	30 ± 1	32 ± 1	30 ± 3
	Nitrogen (%)	4 ± 2	2 ± 1	3 ± 2	2 ± 2	1 ± 1	3 ± 4

Table 2. Total VFA and species concentrations in the sludge substrate and acidogenic reactor at various HRTs.

	Sludge substrate	Acidogenic 2-phase		
HRT (d)		5	3	2
TVFA (mg COD L^{-1})	1,806 ± 501	2,706 ± 546	2,471 ± 258	1,667 ± 410
Acetic acid (mg COD L^{-1})	707 ± 194	83 ± 36	117 ± 25	201 ± 77
Propionic acid (mg COD L^{-1})	513 ± 121	1,382 ± 207	1,330 ± 180	744 ± 256
Isobutyric acid (mg COD L^{-1})	78 ± 19	221 ± 69	181 ± 25	112 ± 29
Butyric acid (mg COD L^{-1})	200 ± 99	246 ± 142	147 ± 36	122 ± 32
Isovaleric acid (mg COD L^{-1})	155 ± 39	469 ± 102	404 ± 52	254 ± 68
Valeric acid (mg COD L^{-1})	117 ± 54	251 ± 93	195 ± 34	153 ± 38

Table 3. Archaeal and bacterial identification of the DGGE band sequences.

Band(s)	Closest related sequence	Order/Class	Max identity (%)
A1-5	*Methanospirillum hungatei* (NR_074177.1)	*Methanomicrobiales*	98-100
A6-10	*Methanolinea mesophila* (AB447467.1)	*Methanomicrobiales*	97-98
A11-12	*Methanoregula formicicum* (NR_102441.1)	*Methanomicrobiales*	97
A13-14	*Methanoculleus receptaculi* (NR_043961.1)	*Methanomicrobiales*	99
A15	*Methanosarcina barkeri* (NR_025303.1)	*Methanosarcinales*	99
B1-2	*Cloacibacterium normanense* (NR_042187.1)	*Flavobacteriales*	100
B3	*Enhydrobacter* sp. (JF792358.1)	*Pseudomonadales*	97
B4	Uncultured *Bacteroidetes* bacterium in sludge AD (CU919567.1)	*Flavobacteriales*	99
B5	Uncultured *Ruminococcaceae* bacterium from wetlands (JX505387.1)	*Clostridiales*	98
B6	Uncultured *Fluviicola* sp. from membrane bioreactor biofilm (GU257757.1)	*Flavobacteriales*	99
B7	*Smithella propionica* (NR_024989.1)	*Syntrophobacterales*	96
B8	Uncultured bacterium in AD treating microcystis bloom (GU559846.1)	*Clostridiales*	89
B9	Uncultured bacterium in AD treating coking wastewater (JQ446286.1)	*Flavobacteriales*	99
B10	*Saccharofermentans acetigenes* (AY949857.1)	*Clostridiales*	98
B11	*Clostridiaceae* bacterium in AD treating cattle waste (AB298771.2)	*Clostridiales*	99
B12	Uncultured bacterium from Evry sludge digester (CT574084.1)	*Flavobacteriales*	97
B13	Uncultured *Firmicutes* bacterium in full-scale AD treating sludge (CU925891.1)	*Clostridiales*	99
B14	Uncultured *Bacteroidetes* in full-scale AD treating sludge (CU925607.1)	*Flavobacteriales*	99
B15	Uncultured *Clostridiales* bacterium treating swine manure (JN173177.1)	*Clostridiales*	97

Figure1

Click here to download high resolution image

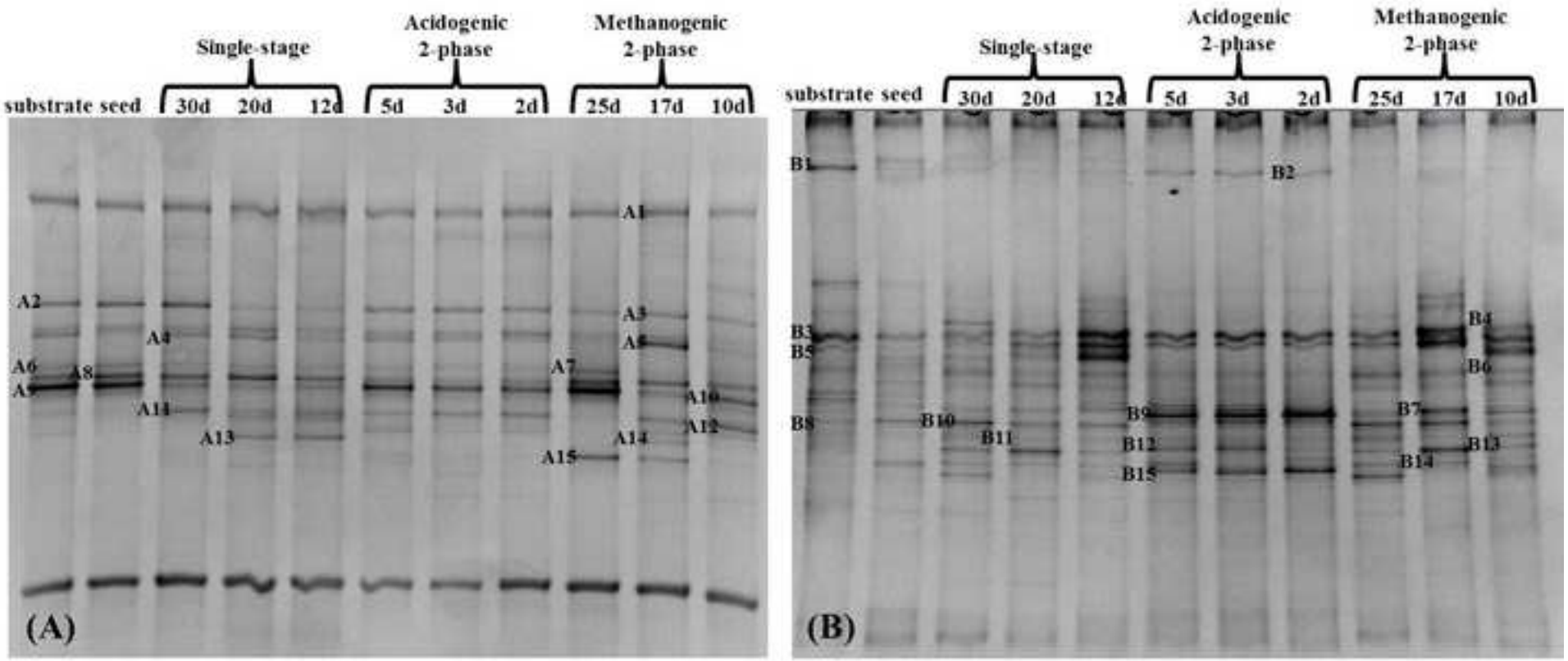


Figure2

Click here to download high resolution image

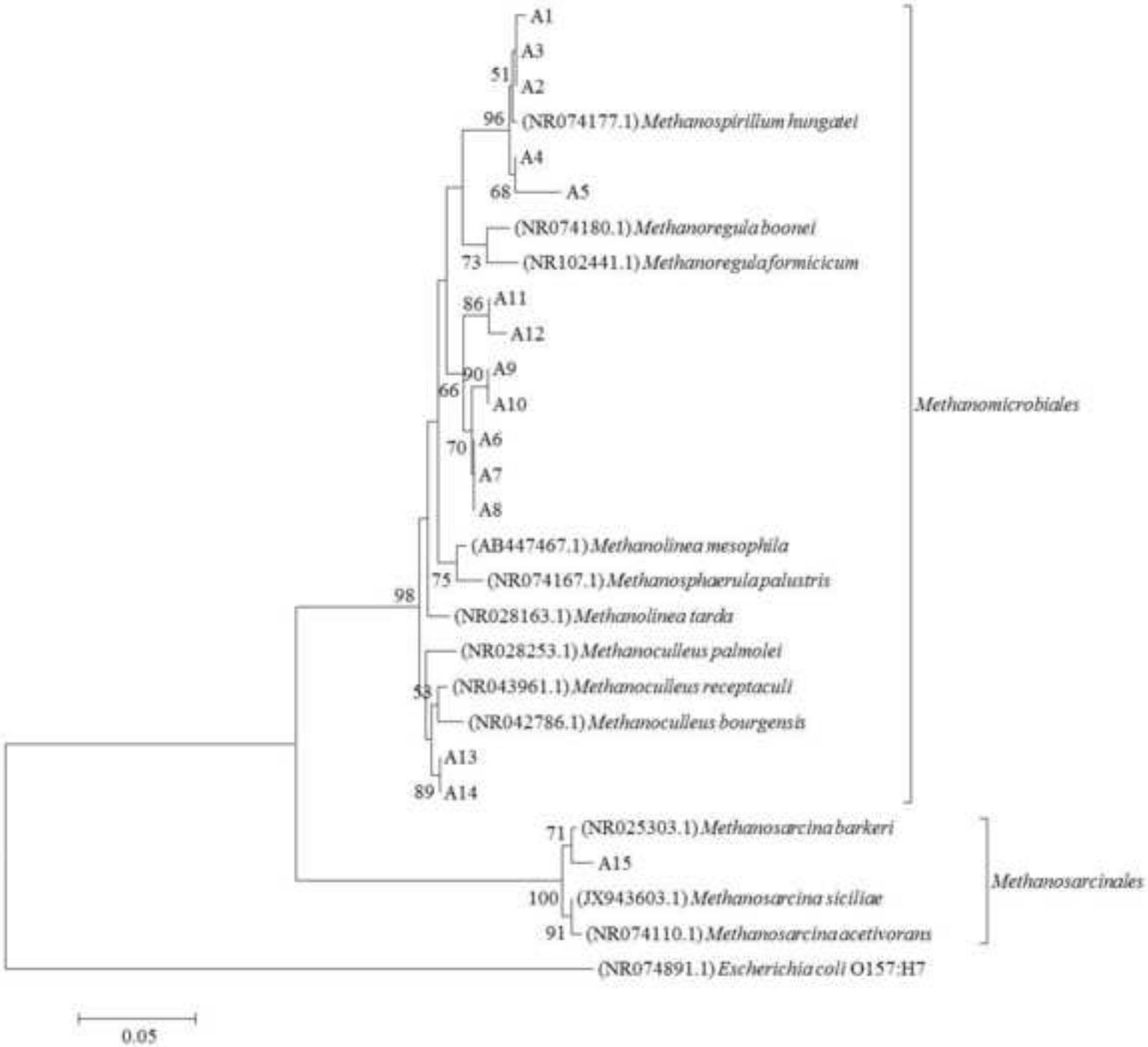


Figure3

Click here to download high resolution image

Figure4aR1

Click here to download high resolution image

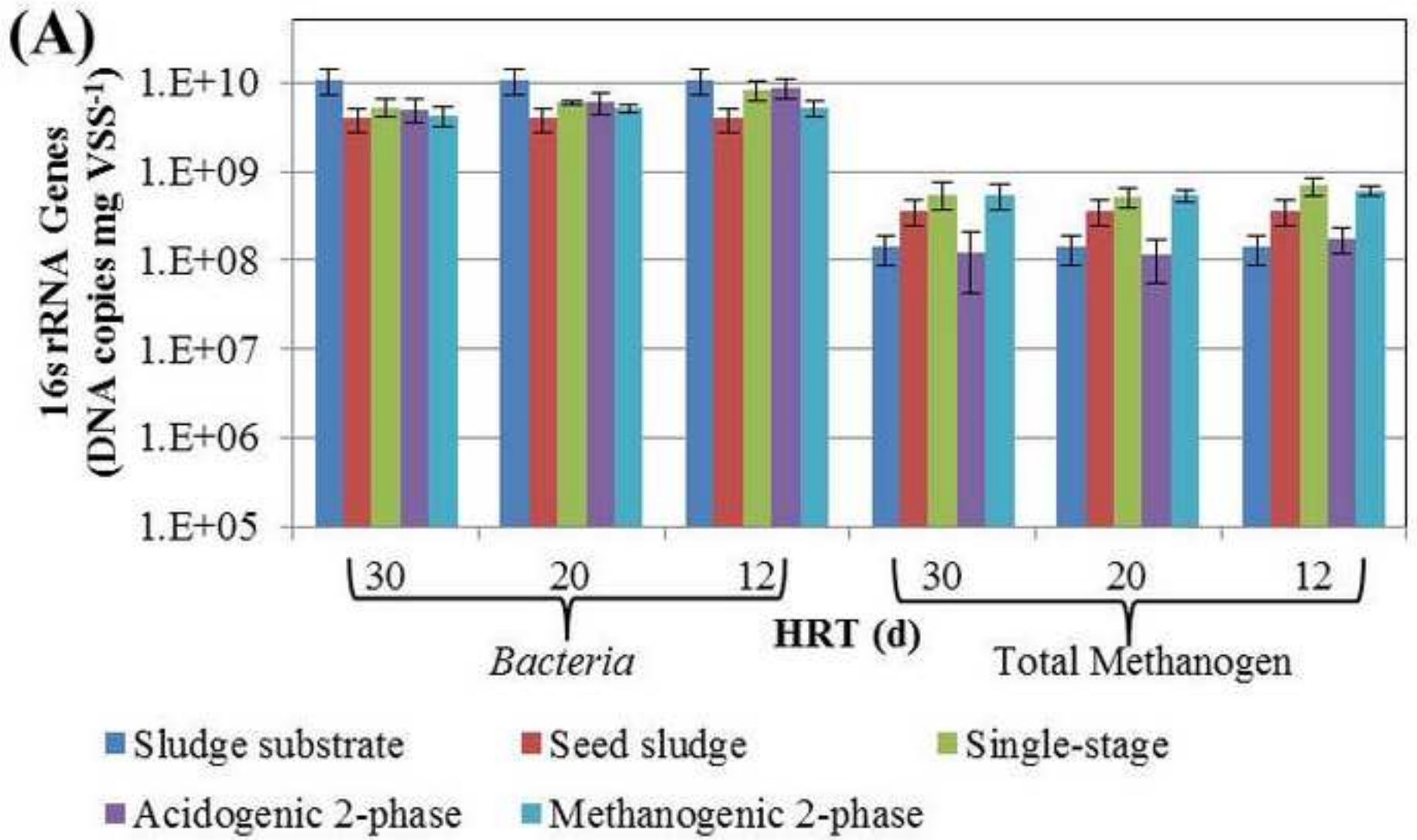


Figure4bR1

Click here to download high resolution image

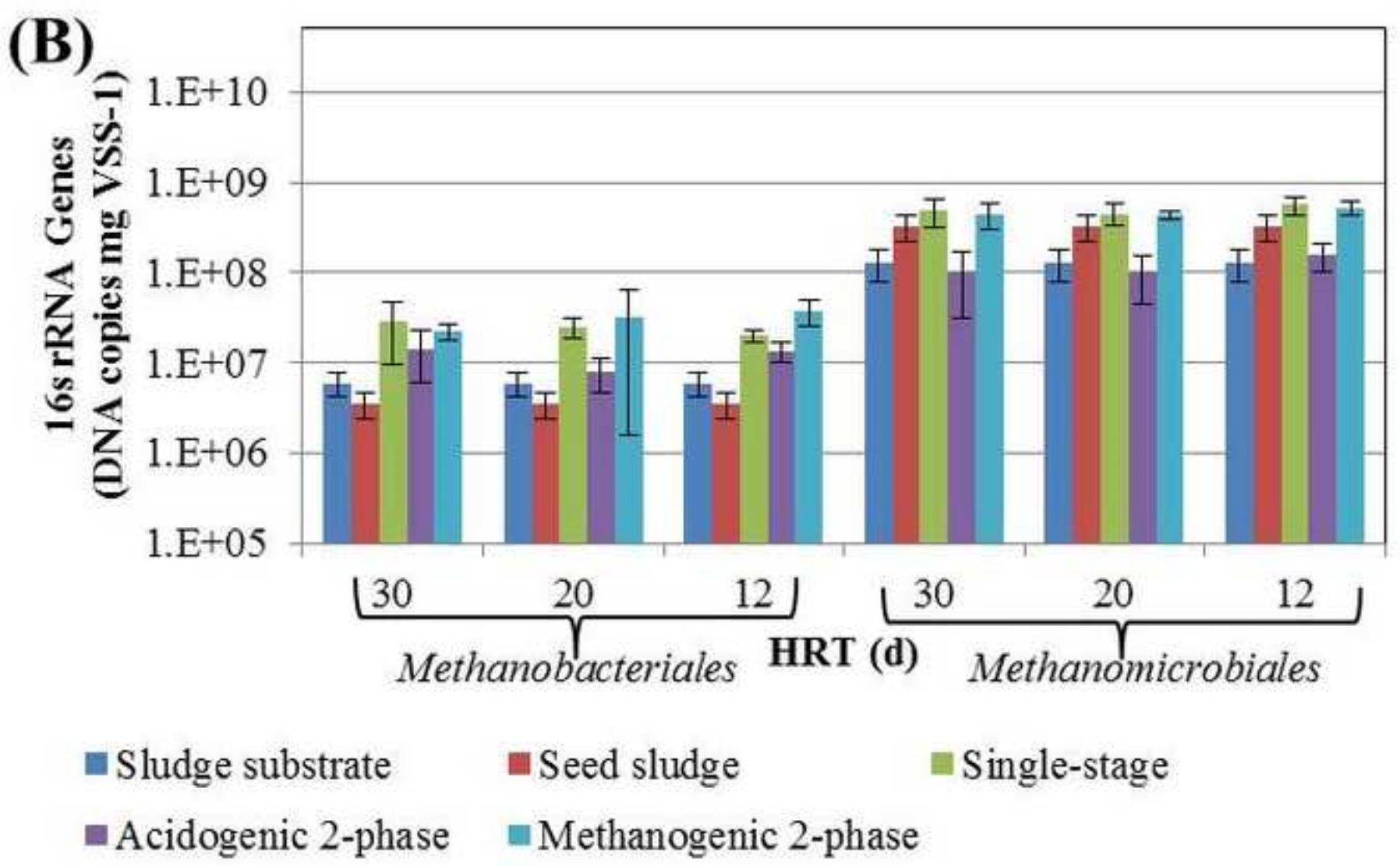
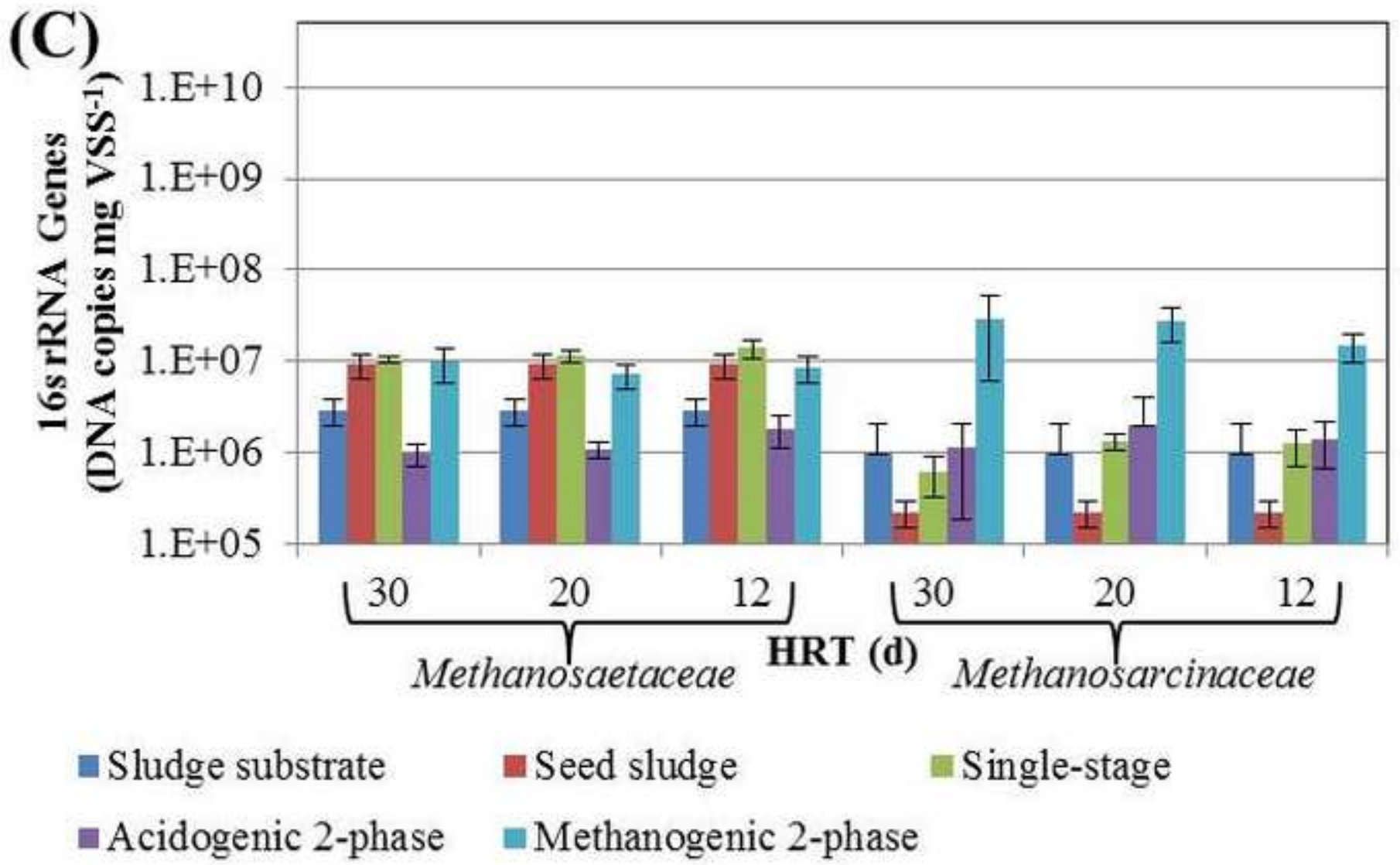


Figure4cR1

Click here to download high resolution image



Tables and Figures Captions

Table 1. Summary of reactor operating parameters and performance in the single-stage and 2-phase system; and in the acidogenic and methanogenic 2-phase reactors.

Table 2. Total VFA and species concentrations in the sludge substrate and acidogenic reactor at various HRTs.

Table 3. Archaeal and bacterial identification of the DGGE band sequences.

Fig. 1. DGGE gel of the (A) archaeal and (B) bacterial population in the sludge substrate, seed sludge and each anaerobic reactor at various HRTs.

Fig. 2. Neighbour-joining tree presenting the archaeal phylogenetic affiliation to the DGGE band sequences.

Fig. 3. Neighbour-joining tree presenting the bacterial phylogenetic affiliation to the DGGE band sequences.

Fig. 4. Quantification of the Bacteria and methanogenic groups in the sludge substrate, seed sludge and anaerobic reactors by qPCR: (A) *Bacteria* and total methanogens; (B) *Methanobacteriales* and *Methanomicrobiales*; (C) *Methanosaetaceae* and *Methanosarcinaceae*.