Pyrosequencing reveals highly diverse microbial communities in microbial electrolysis cells involved in enhanced \( \text{H}_2 \) production from waste activated sludge

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**Abstract**

Renewable \( \text{H}_2 \) production from a plentiful biomass, waste activated sludge (WAS), can be achieved by fermentation, but the yields are low. The use of a microbial electrolysis cell (MEC) can increase the \( \text{H}_2 \) production yields to several times that of fermentation. We have proved that the enhancement of \( \text{H}_2 \) production was due to the ability of MECs to use a wider range of organic matter in WAS than in fermentation. To support this result strongly, we here investigated the microbial community structures of WAS and anode biofilms in WAS-fed MECs. A pyrosequencing analysis based on the bacterial 16S rRNA gene showed that dominant populations in MECs were more diverse than those in WAS (inoculum and substrate) after enrichment, and there was a clear distinction between MECs and WAS in microbial community structure. Diverse acid-producing bacteria and exoelectrogens (predominance of \textit{Geobacter}) were detected in MECs but they were only rarely found in WAS. It has been reported that these acid-producing bacteria can ferment various sugars and amines with acetate, propionate, and butyrate as their major by-products. This was consistent with our chemical analyses. Detected exoelectrogens are known to use these organic acids (mainly acetate) and certain sugars to directly produce current for \( \text{H}_2 \) generation at the cathodes in the MECs. Using quantitative real-time PCR, we demonstrated that a consistent feed of alkaline-pretreated WAS containing large amounts of acetate led to a predominance of acetoclastic methanogens, while hydrogenotrophic methanogens were abundant in MECs fed both raw and alkaline-pretreated WAS. Syntrophic interactions between phylogenetically diverse microbial populations in anodophilic biofilms were found to drive the efficient cascade utilization of organic matter in WAS.

**1. Introduction**

Every year, millions of tons of waste activated sludge (WAS) are produced from biological municipal wastewater processes in countries like China and the United States (Turovskiy and Mathai, 2006). Recently, this plentiful and sustainable biomass residue has been widely used to produce \( \text{H}_2 \), a more valuable energy carrier than \( \text{CH}_4 \), through fermentation (Guo et al., 2010; Zhao et al., 2010). The main drawback of this process is that the \( \text{H}_2 \) yield is often low. One major barrier to
high H₂ yield is that only carbohydrates can be used by microorganisms to produce H₂ in fermentation. In addition, the organic matter in WAS is enveloped in flocs that are not readily bioavailable to the hydrogen-producing bacteria. We have adopted a recent emerging technology, microbial electrolysis cells (MECs), combined with the alkaline pretreatment of WAS to increase H₂ yield from WAS to several times that of fermentation (Lu et al., 2012). MECs were derived from microbial fuel cell (MFC), in which exoelectrogens on the anode oxidize the organic matter to transfer electrons to the anode. Electrons travel through an external circuit to the cathode, where they reduce the protons to produce H₂ at a small applied voltage (about 0.2–0.8 V) (Liu et al., 2005). MECs have also been shown to use volatile fatty acids (VFAs), glucose, cellulose, ethanol, glycerol, protein and some wastewater (e.g. domestic, swine, and fermentation wastewater) for H₂ production (Cheng and Logan, 2007; Ditzig et al., 2007; Lu et al., 2009, 2010; Wagner et al., 2009). Anodophilic reaction process in MECs can be completed through direct oxidation by exoelectrogens or syntrophic interactions among biofilm consortia. Examining the microbial community structures of anodophilic biofilms is quite necessary to understanding the complex interactions occurring on the anode and finding ways to improve the design and operation of scale-up systems.

To date, most studies about the microbial communities in MECs and MFCs have focused on those using single substrates, such as formate, ethanol, acetate, propionate, butyrate, succinate, lactate, glucose, or cellulose (Chae et al., 2009; Cheng et al., 2011; Jung and Regan, 2007; Kiely et al., 2011b; Xing et al., 2009). There are few reports about how practical and complex substrates, such as wastewater and waste, affect microbial community structures, especially for MEC studies. Kiely studied the dynamics of bacterial community by changing operation from MFC to MEC using potato and dairy manure wastewater (Kiely et al., 2011a). Results revealed wide discrepancies between MFC and MEC on the bacterial community; these communities became more diverse than those grown using acetate. Kiely and team also inferred that the poor performances of MFCs/MECs fed dairy manure wastewater were due to the decreased presence of Geobacter in the bacterial community. However, another MFC study using chocolate industry wastewater did not detect any known exoelectrogens but exhibited significantly more power (1500 mW/m²) than that fed dairy manure wastewater (189 mW/m²) (Kiely et al., 2011a; Patil et al., 2009). All of these studies of the microbial community were based on 16S rRNA gene clone libraries.

Previous study suggested that the rare microorganisms in anodophilic biofilm may produce a great deal of power but generally escapes detection by conventional culture-independent approaches such as 16S rRNA gene clone library and DGGE using Sanger sequencing (Kim et al., 2010). These molecular biology methods lack sufficient sequences to capture comprehensive and systematic information on diverse microbial communities. The two most comprehensive microbial community surveys for MFCs have sampled 300 and 461 clones, respectively, but no more than 42 phylotypes were detected in each sample based on a sequence similarity of >97% (Ishii et al., 2008; Patil et al., 2009). A new highly parallel sequencing technique 454 GS-FLX pyrosequencing, can generate 400,000 sequences (100–400 bp in length), compared with the 192 sequences (700 bp) produced by one run of Sanger sequencing (Zhang et al., 2009). Two studies used it for bacterial diversity surveys in MFCs/MECs and obtained a total of 31,901–94,284 reads (Lee et al., 2010; Parameswaran et al., 2010). These studies reported thousands of phylotypes and found that numerous bacteria in the anodophilic biofilms were novel. Less abundant bacteria were generally associated with electricity generation. Pyrosequencing can provide significant insight into the microbial community structures in bioelectrochemical systems.

In our previous MEC study using WAS, a cascade utilization of various organic substances by anodophilic biofilm was proved to be responsible for enhancement of H₂ yield from WAS through a balance calculation (Lu et al., 2012). However, little is known about how complex syntrophic interactions occur on the anodes of MECs. These interactions should explain how MECs make use of a wider range of substrates than fermentation does. We need to know the effect of alkaline pretreatment on the composition of the microbial communities in MECs. In order to accomplish these goals, we here examined bacterial community structures using 454 GS-FLX pyrosequencing of the 16S rRNA gene in much greater depth. The quantities of bacteria, Archaea, and methanogens (hydrogenotrophic and acetoclastic) were examined by quantitative real-time PCR (QPCR).

2. Materials and methods

2.1. MEC construction and operation

Two-chamber MECs (TMECs) were fixed with a graphite brush anode (5 cm diameter × 7 cm length) and a carbon cloth cathode (7 cm²) containing 0.5 mg/cm² Pt catalyst. Two chambers were separated by a cation exchange membrane (4.9 cm²). The anode and cathode chambers were filled with 250 mL of WAS solution and phosphate buffer solution (PBS) (Na₂HPO₄, 9.16 g/L; NaH₂PO₄, 4.9 g/L; NH₄Cl, 0.31 g/L; KCl, 0.13 g/L), respectively. The WAS solutions were prepared by mixing raw WAS (R-WAS) or alkaline-pretreated WAS (A-WAS, at constant pH 10 over 8 days) with PBS. The characteristics of WAS solutions were as follows: volatile suspended solids (VSS) 5.01 ± 0.77 (R-WAS) and 2.33 ± 0.17 (A-WAS) mg/L, total chemical oxygen demand (TCOD) 5980 ± 442 (R-WAS) and 4071 ± 611 (A-WAS) mg/L, and soluble chemical oxygen demand (SCOD) 396 ± 159 (R-WAS) and 2474 ± 142 (A-WAS) mg/L. Single-chamber MECs (SMECs) were constructed as described previously and were fed with only A-WAS solution (Lu et al., 2009). The MEC anodes were enriched in MFC equivalents and then transferred to MECs. The operation of the MECs and the methods for assessing reactor performance were performed as described by Lu et al. (Lu et al., 2012). Anodes were exposed to air for 15 min at every interval between the cycles to inhibit the growth of methanogens but gave a slight influence to anaerobic bacteria involved in MECs (Call and Logan, 2008; Lu et al., 2009).

2.2. DNA extraction

After two months of operation, graphite fibers were cut from anodes and fragmented using sterile scissors. Graphite fibers
were sampled from the top, middle and bottom sections of a brush. Three parts of fibers were combined for DNA extraction. Before DNA extraction the fibers were gently rinsed with deionized water to remove the residual sludge. One milliliter R-WAS was centrifuged at 8000g to supernatant, about 0.25 g pellet was used for DNA extraction. Total genomic DNA was extracted from fibers and R-WAS using a PowerSoil DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA) according to the manufacturer’s instructions. The quantity and quality of the extracted DNA were checked by measuring its absorbance at 260 and 280 nm using a Beckman DU800 spectrophotometer.

### 2.3 High-throughput 16S rRNA gene pyrosequencing

Amplicon libraries were constructed for 454 pyrosequencing using bacterial primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 533R (5′-TTACCAGCMGCTGCTGACAC-3′) for the V1-V3 region of the 16S rRNA gene. The fused forward primer includes a 10-nucleotide barcode inserted between the Life Sciences primer A and the 8F primer. The barcodes were used to sort multiple samples in a single 454 GS-FLX run. After being purified and quantified, a mixture of amplicons was used for pyrosequencing on a Roche massively parallel 454 GS-FLX according to standard protocols (Margulies et al., 2005). Raw pyrosequencing data that obtained from this study were deposited to the NCBI Sequence Read Archive (SRA, http://trace.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?) with accession no. SRA047640. To minimize the effects of random sequencing errors, low-quality sequences were removed by eliminating those that without an exact match to the forward primer, without a recognizable reverse primer, length shorter than 200 nucleotides and contained any ambiguous base calls (N). We trimmed the barcodes and primers from the resulting sequences. Finally, pyrosequencing produced 10,743 (TMEC), 9374 (SMEC) and 8164 (R-WAS) high-quality V1-V3 tags of the 16S rRNA gene with an average length of 455 bp.

### 2.4 Biodiversity analysis and phylogenetic classification

We clustered sequences into operational taxonomic units (OTUs) by setting a 0.03 or 0.05 distance limit (equivalent to 97% or 95% similarity) using the MOTHUR program. Rarefaction curves, Shannon diversity index (http://www.mothur.org/wiki/Shannon) and species richness estimator of Chao1 (http://www.mothur.org/wiki/Chao) were generated in MOTHUR for each sample. Sequences were phylogenetically assigned to taxonomic classifications using an RDP naïve Bayesian rRNA classifier with a confidence threshold of 80% (http://rdp.cme.msu.edu/classifier/classifier.jsp). After phylogenetic allocation of the sequences down to the phylum, class and genus level, relative abundance of a given phylogenetic group was set as the number of sequences affiliated with that group divided by the total number of sequences per sample. Hierarchical cluster analysis was performed using gplots package of R (http://www.r-project.org/) in Linux. Principal component analysis (PCA) was conducted by MOTHUR. A Venn diagram with shared and unique OTUs was used to depict the similarity and difference between the three communities.

### 2.5 Quantitative real-time PCR

We used TaqMan based real-time PCR to quantify total bacteria, Archaea, two hydrogenotrophic methanogen orders Methanobacterales (MBT) and Methanomicrobiales (MMB), and two acetoclastic methanogen families Methanosarcinaceae (MSC) and Methanosetaeaceae (MST) within the order Methanosarcinales. These three orders have been found to cover most methanogens in bioelectrochemical systems (BES) (Chae et al., 2010; Lee et al., 2009; Parameswaran et al., 2010). Quantitative real-time PCR was performed using the ABI 7500 FAST system with the corresponding primer and probe sets (Yu et al., 2005) listed in Supplementary Table S1. A 20 μL real-time PCR mixture contained 10 μL of the Premix Ex Taq (Probe qPCR) (TaKaRa Bio Inc., Japan), 0.4 μL of each primer (0.2 μM final concentration), 0.8 μL of TaqMan probe (0.2 μM final concentration), 0.4 μL of ROX Reference Dye II, 2 μL of template DNA, and 6 μL of PCR-grade pure H2O. QPCR was performed in a two-step amplification procedure: initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 40 s.

Standard curves were generated as described previously (Yu et al., 2006) using the representative strains listed in Table S2. Five strains were purchased from German Collection of Microorganisms and Cell Cultures and American Type Culture Collection (ATCC). Genomic DNA was extracted from each strain and the target 16S rRNA gene sequences were amplified by conventional PCR using the corresponding primers listed in Table S1. Amplicons were cloned into pMD 19-T Vector (TaKaRa Bio Inc., Japan). Each plasmid was serially diluted with a 10-fold step ranging from 10^2 to 10^8 copies/μL and directly used as template for QPCR with the corresponding primer and probe sets. The threshold cycle (CT) values determined were plotted against the logarithm of initial copy concentrations. Characteristics of the QPCR standard curves can be found in Table S2. These were up to the statistical error requirements (Yu et al., 2006). Each reaction (standard curve and sample) was conducted in triplicate.

### 3. Results

#### 3.1 Enhanced H2 production from WAS using MECs

The use of two-chamber MECs (TMECs) for H2 production from raw waste activated sludge (R-WAS) produced an average H2 production yield of 3.89 ± 0.39 mg-H2/g-DS, which was much larger than that of fermentation of WAS with or without pretreatment (ranged from 0.02 mg-H2/g-DS to 1.8 mg-H2/g-DS) (Lu et al., 2012). When an alkaline-pretreated waste activated sludge (A-WAS) was fed into the TMECs, the yield almost doubled due to the production of many types of bioavailable organic matter by pretreatment. Single-chamber MECs (SMECs) with low internal resistance showed a H2 production rate 13 times that of TMECs with similar H2 yield using A-WAS, but CH4 was detected in the biogas (Lu et al., 2012). A certain amount of acetate, propionate, and butyrate was detected in A-WAS and the process of electrohydrogenesis. A balance calculation based on thermodynamic H2 yield showed that the carbohydrate and its fermentative by-
products, organic acids, were not the sole fuels for MECs. Rather, other organic matter, such as protein, may have been directly or indirectly used for H₂ production. Cascade utilization of a wider range of organic matter in WAS was found to be responsible for the most of the increased yield in MECs relative to fermentation.

3.2. Richness and diversity of bacteria phylotypes

Three 16S rRNA gene libraries were constructed from pyrosequencing of TMEC, SMEC, and R-WAS communities with 10,743, 9374 and 8164 high-quality reads (average length of 455 bp), respectively. We obtained 1484 (SMEC), 1359 (TMEC) and 1291 (R-WAS) operational taxonomic units (OTUs) at a 3% distance. However, new bacterial phylotypes continued to emerge even after 10,000 reads sampling with pyrosequencing (Fig. 1). The total number of OTUs estimated by Chao1 estimator were 2802 (SMEC), 2336 (TMEC), and 2306 (R-WAS) with infinite sampling, indicating that SMEC had the greatest richness than TMEC and R-WAS. These results suggest that pyrosequencing revealed bacterial communities in MECs to be more diverse than those in the most comprehensive surveys previously conducted using complex substrates on the basis of Sanger sequencing (Ishii et al., 2008; Patil et al., 2009). The Shannon diversity index provides not only the simply species richness (i.e., the number of species present) but how the abundance of each species is distributed (the evenness of the species) among all the species in the community. SMECs had the highest diversity (Shannon = 5.82) among the three communities. The Shannon index of TMEC (5.52) was slightly larger than 5.36 in R-WAS.

3.3. Comparative analysis of bacterial communities

Hierarchical cluster analysis was used to identify the differences of three bacterial community structures (Fig. 2). There were two clusters. The MEC group (including TMEC and SMEC) was separated from the R-WAS, suggesting clear distinctions of community structure between the anode biofilm and R-WAS despite the fact that they shared the same source of microbial consortia. This was supported by the principal component analysis (PCA) (Fig. 3). Principal components 1 and 2 explained 59.3% and 27.7% of the total community variations, respectively. Two MEC samples were clustered together.
and were well separated from that from R-WAS, but there was a clear distinction between TMEC and SMEC. These results show that particular bacteria selectively enriched on the MEC anodes, and different reactor configuration had an obvious effect on the community structures.

The sum of total observed OTUs in all three communities was 3277, but only 187 OTUs or 5.7% of the total OTUs were shared by them (Fig. 4). The majority (74.4%) of the shared OTUs were Proteobacteria and Bacteroidetes. TMEC and SMEC had more common OTUs (425, 13% of total) than any of them with R-WAS (TMEC/R-WAS, 244, 7%; SMEC/R-WAS, 375, 11%). OTUs that were unique to each community numbered 877 (TMEC), 871 (SMEC) and 859 (R-WAS), and together they accounted for 80% of the total number of observed OTUs.

3.4. Bacterial taxonomic identification

To identify the phylogenetic diversity of bacterial communities in MECs and R-WAS, we assigned qualified reads to known phyla, classes and genera (Fig. 5). Three communities showed an extremely high diversity, reflected in the fact that 21 (TMEC), 18 (SMEC) and 19 (R-WAS) identified bacterial phyla were detected (Fig. 5a). In total, 25 identified phyla were observed. Even so, 8.8% (TMEC), 9.9% (SMEC), and 6.9% (R-WAS) of the total reads were unclassified bacteria at a class level increase to 27.1% (TMEC), 27.4% (SMEC), and 16.9% (R-WAS) of the total reads.

Fig. 3 – Principal component analysis (PCA) of bacterial communities from TMEC, SMEC and R-WAS based on pyrosequencing of 16S rRNA gene.
The bacteria (Zoogloea 3.9%, Haliscomenobacter 3.7%, Nitrospira 3.4%, and Dechloromonas 3.3%) involved in wastewater treatment were the most abundant populations in R-WAS. A genus of Ferrribacterium can reduce Fe(III) with organic acids as electron donors (Cummings et al., 1999). Its presence in R-WAS (1.4%) could be attributed to a high ferric iron concentration (20.71 mg/g-DS) in R-WAS. However, this Fe(III)-reducing bacterium cannot use electrodes as electrons acceptors. For this reason, it absent from the MECs.

Diverse known exoelectrogens were detected by pyrosequencing in MECs (Table 1), but they were hardly found in R-WAS. Geobacter was the most dominant population. Other species capable of generating electricity were not numerically abundant and only accounted for averaged 0.28% (0.01–1.94%) of the total composition in MECs. One typical exoelectrogen, Shevanella, was not found in MECs. This was consistent with the observation that no lactic acid, a major substrate of Shevanella, was detected during the tests. Our results suggest that it would be very hard to study exoelectrogens with extremely low abundance in microbial communities using culture or any low-throughput molecular method unless a pyrosequencing was also used.

3.5. Composition of the Archaea community

The numbers of copies of the Archaea 16S rRNA gene was 3.9 × 10^3 copies/ng-DNA (TMEC), 1.6 × 10^3 copies/ng-DNA (SMEC), and 9.9 × 10^2 copies/ng-DNA (R-WAS), accounting for 0.82% (TMEC), 0.27% (SMEC), and 0.07% (R-WAS) of the total bacteria 16S rRNA genes, respectively (Fig. 6). Two hydrogenotrophic methanogen orders Methanobacteriales (MBT) and Methanomicrobiales (MMB), and two acetoclastic methanogen families Methanosarcinaceae (MSC) and Methanosetaeaceae (MST) were all detected in three microbial communities. These methanogens cover almost all the Archaea population in each microbial community. Hydrogenotrophic methanogens dominated the TMEC’s Archaea population and accounted for 63.8% of the total Archaea. In SMEC, the dominant Archaea population shifted to the acetoclastic methanogens constituting 51.7% of the total Archaea.

4. Discussion

Microbial communities in MECs using WAS showed greater diversity than those in R-WAS and MFCs fed fermentation products (formic, acetic, lactic, succinic acids, and ethanol) (Kiely et al., 2011b). Now, more studies support the view that greater biodiversity increases ecological stability (Tilman et al., 2006; Wrighton et al., 2010). Community evenness has been found to be key factor to resisting environmental stress (Wittebolle et al., 2009). WAS is a complex organic matter with a limited number of bioavailable organic compounds. The high diversity of anodophilic microbial communities in WAS-fed MECs can be considered as a response of biofilms against the substrate-restricted environment. Another point that supports this is a recent study that showed that a shift of acetate-fed MFCs from a copiotrophic to an oligotrophic environment significantly increased the diversity of their communities (Lee et al., 2010).

The bacterial community structures in WAS-fed MECs are similar to that obtained using a clone library for one anaerobic fermentation system used for enhancement of VFAs production from protein in WAS (Feng et al., 2009). In this fermentation system, Clostridia (45.6%), β-Proteobacteria (21.1%), Bacteroidia (11.4%), γ-Proteobacteria (4.5%), α-Proteobacteria (2.6%), and Sphingobacteria (2.6%) formed a dominant community structure with acetic and propionic acid as its major acidification products. The congruence of two studies suggests a similar functional role of both microbial communities in conversion of carbohydrate and protein to organic acids. An exception is that abundant δ-Proteobacteria was found in MECs compared to its absence from the fermentation system. This is because most exoelectrogens (e.g., Geobacter) in MECs belong to δ-Proteobacteria. An analysis of bacterial community structure at the genus level also showed that fermentative bacteria, which can produce organic acids from carbohydrates and protein, were abundant in MECs. Effective conversion of the carbohydrates and proteins contained in WAS to bioavailable organic matter capable of being used by exoelectrogens was responsible for high yields obtained in MECs using WAS.

Diverse exoelectrogens, which vary in electron donors and pathways of extracellular electron transfer, were found in
MECs. Besides the species Geobacter, other well-known exoelectrogens only accounted for small fractions in bacterial community. There could have been new exoelectrogens in communities because the electricity production ability of many unclassified bacteria was not yet known. However, they might have contribution to electricity generation in MECs. Using glucose as an electron donor, Klebsiella pneumoniae (Zhang et al., 2008) and Rhodoferax ferrireducens (Chaudhuri and Lovley, 2003) have been found to directly transfer electrons to the electrode, and Alcaligenes faecalis by excreting the redox mediators to produce electricity in MFC (Rabaey et al., 2004). Arrobacter butzleri (Fedorovich et al., 2009), denitrifying bacteria C. denitrificans (Xing et al., 2010), sulfate-reducing bacteria Desulfuromonas acetoxidans (Bond et al., 2002), and Geothrix fermentans (Bond and Lovley, 2005) can oxidize various organic acids (e.g. acetate, propionate, lactate, and succinate) and use electrode as a electron acceptor directly. Another sulfate-reducing bacterium Desulfovibrio desulfuricans capable of utilizing natural sulfate/sulfide mediator to generate electricity from lactate in MFC (Ieropoulos et al., 2005).

Methanogens were enriched on the anodes of MECs with number an order of magnitude higher than that observed in R-WAS. This was due to the anaerobic environment of the MECs. A different operating process used for the two reactors might be why more methanogens were found in TMEC than in SMEC. Although anodes were exposed to air for 15 min at
every interval between the cycles to inhibit the growth of methanogens, methanogens were more prone to grow in TMECs because they had a longer batch-cycle time (140 – 210 h) than that in SMECs (11 – 15 h) (Fig. S2). The predominance of hydrogenotrophic methanogens in TMECs suggested that H2 might come from fermentation of carbohydrates or diffusion from the cathode through an ion exchange membrane. The major organic acid containing in R-WAS was acetate. This explained the dominance of Methanosaeta in SMEC. Methanosaeta has a lower half-maximum-rate concentration ($K_s < 5$ mg/L) than another acetate-utilizing methanogen, Methanosarcina, for acetate (Jetten et al., 1992). Relatively few H2-oxidizing methanogens were detected in SMEC, suggesting that a H2 sink due to the methanogenesis was controlled effectively. However, additional H2 purification was needed in SMEC. Methane production in MECs consumes H2 directly or causes a loss of electron donors. In order to further increase the H2 yield from WAS, more effective and economical approaches for inhibition of methanogenesis in MECs, such as reactors that can be operated at relative low temperatures, are necessary (Lu et al., 2011).

## 5. Conclusions

Using high-throughput pyrosequencing, we demonstrated that microbial populations in MECs became more diverse than WAS after being enriched and fed by WAS. Two bacterial groups of fermentative acidogenic bacteria and exoelectrogens with diverse species and wide range of abundance were largely found in anodophilic biofilms of MECs, but were mostly absent in WAS. Syntrophic interactions between the two bacterial groups drove MECs to cascade utilization of various organic matter including carbohydrates, proteins, and other substances, for H2 production. Using QPCR, we showed that a consistent feed of alkaline-pretreated WAS containing large amounts of acetate was responsible for the predominance of acetoclastic methanogens, while hydrogenotrophic methanogens were abundant in TMECs fed both raw and alkaline-pretreated WAS. Our results significantly support previous findings that more kinds of substrate can be used by bacteria in MECs than those undergoing fermentation and that this can increase H2 production yield from WAS in view of molecular ecology.

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## Appendix. Supplementary material

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.watres.2012.02.005.

## References


