

Article

Riboflavin-Functionalized Conductive Material Enhances a Pilot-Scaled Anaerobic Digester Fed with Cattle Manure Wastewater: Synergies on Methanogenesis and *Methanosarcina barkeri* Enrichment

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Abstract

Anaerobic digestion (AD) technology is universally acknowledged as the most economically viable and efficient approach for energy recovery from livestock manure. To validate the efficacy of riboflavin-functionalized carbon-based conductive materials (CCM-RF) in enhancing methane production at pilot scale, three pilot-scale upflow anaerobic sludge blanket (UASB) reactors were constructed and separately supplemented with carbon cloth (CC), granular activated carbon (GAC), and a combination of CC and GAC. During reactor initialization, riboflavin and a concentrated inoculum of *Methanosarcina barkeri* (*M. barkeri*) were introduced to investigate the mechanistic role of CCM-RF in promoting direct interspecies electron transfer (DIET) and optimizing treatment efficiency during anaerobic digestion of cattle manure wastewater. The results showed that all reactors improved AD performance and maintained stable operation at the OLR of 15.66 ± 1.95 kg COD/(m³·d), with a maximum OLR of 20 kg COD/(m³·d) and the HRT as short as 5 days. Among the configurations, the CC reactor outperformed the others, achieving a methane volumetric yield of 6.42 m³/(m³·d), which represents an eight-fold increase compared to conventional AD systems. Microbial community analysis revealed that, although *M. barkeri* was initially inoculated in large quantities, *Methanobrevibacter*—a methanogen with DIET capability—eventually became the dominant species. The enrichment of *Methanobrevibacter* and the simultaneous enhancement in sludge conductivity collectively verified the mechanistic role of CCM-RF in promoting CO₂-reductive methanogenesis through strengthened DIET pathways. Notably, *M. barkeri* showed progressive proliferation under conditions of high organic loading rates (OLR) and short hydraulic retention time (HRT). This phenomenon provides a critical theoretical basis for the development of future strategies aimed at the targeted enrichment of *Methanosarcina*-dominant microbial consortia.

Keywords: cattle manure wastewater; anaerobic digestion; carbon-based conductive materials with riboflavin; DIET; 16sRNA



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1. Introduction

With the growing global focus on environmental protection and sustainable development, the efficient energy recovery and resource utilization of livestock manure have emerged as pivotal factors for advancing the high-quality development of the livestock breeding industry. According to statistics from the Food and Agriculture Organization of the United Nations, the global cattle population is approximately 1.5 billion head [1]. Based on biomass and metabolic rate models, cattle manure is estimated to account for approximately 65–70% of total livestock feces [2]. As a core pollution source in livestock farming, cattle manure accounts for 60–70% of global agricultural non-point source pollution, thereby becoming the primary target for remediation in the sustainable development of the livestock sector. This holds significant implications for global carbon emission mitigation and the mission of “Establishment of Zero-Pollution Earth” [3–5].

Under the backdrop of diminishing fossil fuel reserves, the resource-efficient utilization of livestock and poultry manure via AD technology has emerged as a critical strategy for enhancing carbon recovery and advancing biomass energy production. In AD processes, interspecies electron transfer (IET) between acidogenic bacteria and methanogenic archaea is a vital step in syntrophic methane production, typically achieved through interspecies hydrogen transfer or formate transfer [6]. However, the imbalance between rapid hydrolysis/ acidogenesis and slower methanogenesis restricts the treatment capacity to relatively low efficiency, thereby limiting methane productivity [7]. Once the AD system becomes unstable, restarting the system often requires a prolonged recovery period, posing significant environmental and economic risks to the associated livestock enterprises. In recent years, the discovery of DIET has provided a potential breakthrough for overcoming the rate-limiting electron transfer processes [8,9]. Consequently, AD treatment technology utilizing conductive materials to enhance the DIET methanogenic pathway for livestock manure has emerged as an effective approach and a research hotspot for improving the resource utilization of livestock waste.

The addition of exogenous conductive materials (e.g., GAC, CC, biochar, graphene, magnetite, and polyaniline) to AD systems enhances the participation of methanogenic archaea (e.g., *M. barkeri*) in DIET, thereby improving methane conversion rate during AD processes [10–12]. Exogenous conductive materials possess high electrical conductivity, chemical stability, and strong adsorption capacity, and thus serve as critical supplements to biological electrical connections [13]. Furthermore, they eliminate the energy cost associated with the microbial synthesis of endogenous conductive structures, enabling microbes to allocate more energy to metabolic activities. With advancements in research on interspecies electron transfer within microbial communities during AD, it has been found that many bacteria secrete riboflavin as a redox mediator, which plays a pivotal role in electron exchange between methanogenic archaea and their syntrophic partners [14]. Riboflavin acts dualistically as an enzymatic cofactor and an electron shuttle, accelerating both biochemical metabolism and electron transfer rates during methanogenesis [15]. The coupling of exogenous conductive materials with riboflavin provides a synergistic strategy for the targeted enhancement of methanogenic efficiency in AD systems. This approach offers a promising and adaptable solution for the high-efficiency resource utilization of livestock and poultry manure.

In previous studies, the application of CCM-RF at the laboratory scale increased methane production efficiency by 37% during the AD of cattle manure wastewater [16]. Specifically, the CC significantly enriched DIET-capable methanogens such as *Methanothrix*, while the riboflavin coating further promoted the enrichment of electroactive bacteria such as *Brooklawnia* and *Anaerolinea*, which are associated with extracellular electron transfer (EET). Additionally, this strategy notably enhanced the activity of enzymes involved

in the CO₂-reductive methanogenesis pathway and increased the abundance of DIET-related functional genes (e.g., *fpoD* and *hdrA*). However, pilot-scale studies that validate the combined effects of CCM-RF on improving methane production performance in AD systems remain scarce. Furthermore, research investigating the response of microbial community structure and the underlying metabolic mechanisms regulated by methanogenic archaea in such systems is still limited.

This study aimed to evaluate the synergistic effects of different CCM-RF on methane production enhancement in pilot-scale reactors, while systematically screening for optimal exogenous conductive materials. Additionally, high-dose inoculation of *M. barkeri* during reactor startup was implemented to assess its potential in accelerating methanogen enrichment and reducing the system acclimatization period. Microbial community analysis was systematically conducted to elucidate the impacts of *M. barkeri* inoculation as an external intervention on DIET-mediated metabolic mechanisms during AD of cattle manure wastewater. From the dual perspectives of performance efficiency and economic viability, this study investigates the applicability of CCM-RF technology for treating high-strength cattle manure wastewater.

2. Materials and Methods

2.1. Experimental Materials

The cattle manure wastewater used in this study was sourced from a large-scale dairy farm in Shaanxi Province, China, which generates 800–1000 m³/d of wastewater primarily composed of dairy cow excreta, barn washing wastewater, and milking parlor cleaning wastewater. Anaerobic granular sludge was purchased from Liboyuan Environmental Materials Co., Ltd. (Weifang, China). The characteristics of the cattle manure wastewater and anaerobic sludge are summarized in Table 1. CC, GAC, and riboflavin were purchased from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). *M. barkeri* was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). *M. barkeri* was enriched and cultured in a temperature-controlled incubator (DHP-9052, Zhongkeboda Co., Ltd., Beijing, China) under anaerobic conditions (N₂:CO₂, 80:20, v/v) using a modified DSM 120 medium containing 30 mM acetate. The medium was modified to include 0.5 mM sulfide, 1 mM cysteine, 0.002 g/L CaCl₂·2H₂O, 1 g/L NaCl, 2 g/L NaHCO₃, and excluded yeast extract, Casitone, and resazurin [5,17].

Table 1. The characteristics of cattle manure wastewater and anaerobic sludge.

Parameter	Cattle Manure Wastewater	Anaerobic Sludge
Total solids–TS (%)	5.78 ± 0.59	1.20 ± 0.08
Volatile solids–VS (%)	3.98 ± 0.53	1.11 ± 0.10
Water content (%)	94.22 ± 0.58	98.20 ± 0.11
pH	8.12 ± 0.11	7.23 ± 0.03
Total chemical oxygen demand–tCOD (g/L)	88.76 ± 13.23	—
C/N	23.45	—
H (%)	6.46 ± 0.45	—
O (%)	33.56 ± 1.45	—

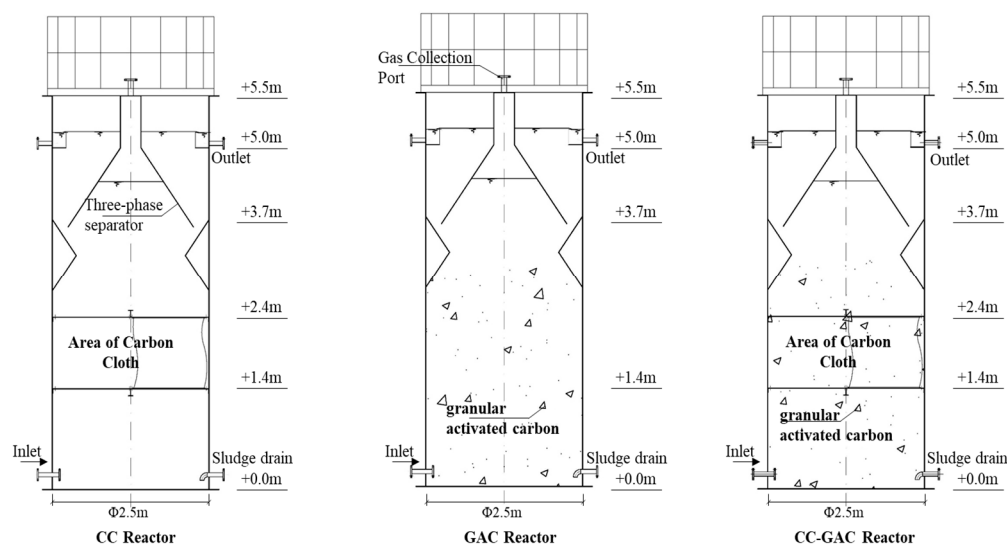
2.2. Experimental Design and Operation

The UASB reactors were constructed in the wastewater treatment area of the dairy farm under pilot-scale conditions (Figure 1a). The working volume of each reactor is 20 m³ (Φ2.4 m × 5.5 m). All reactors were operated under mesophilic anaerobic conditions with a controlled temperature of 30 °C. The UASB reactors were separately supplemented with CC, GAC, and a combination of CC and GAC (Figure 1b). For CC-supplemented

reactors, the CC was secured with nylon ropes within the 1.4–2.4 m elevation above the reactor bottom to maintain suspension and ensure uniform mixing with anaerobic sludge throughout the treatment system. For GAC-supplemented reactors, GAC (particle size: 2–4 mm) was co-inoculated with anaerobic sludge at a dosage of 2% (w/w).



(a)



(b)

Figure 1. (a) On site as-built diagram of pilot plant; (b) reactor design drawing.

During the initial operational phase, 4 m³ of anaerobic granular sludge, 5 kg of riboflavin, and 10 L of concentrated *M. barkeri* (with a volatile suspended solids concentration of 4.5 g/L) inoculum were introduced into each reactor. During reactor operation, the HRT was reduced in a stepwise manner. Each phase was maintained until COD removal efficiency and biogas production stabilized, after which the feeding rate was increased to elevate the OLR in incremental steps not exceeding 50% of the previous value (Table 2). The total operational duration of the experimental system was approximately 120 days.

A recirculation pipeline installed at the reactor top allowed a circulation pump to transfer upper-phase material back to the inlet, achieving internal homogenization of the system. Daily feeding and sampling were performed at fixed intervals. Biogas yield, effluent COD concentration, and pH were measured to comparatively analyze the enhancement effects of different CCM-RF on the AD of cattle manure wastewater.

Table 2. The HRT and the OLR of the feed in each reactor.

HRT	Average tCOD (g/L)	OLR (kg COD/(m ³ ·d))
40 days	75.38	1.63 ± 0.18
20 days	71.01	3.76 ± 0.74
13.3 days	69.69	5.51 ± 1.02
10 days	53.51	7.10 ± 0.94
8 days	53.15	6.80 ± 0.84
6.7 days	70.35	7.94 ± 0.53
5 days	74.61	13.73 ± 2.06
4 days	75.73	18.57 ± 2.66
5 days	79.78	15.66 ± 1.94

2.3. Standard Parametric Detection Methods

All analytical parameters, including COD, TS, and VS, were determined following Standard Methods for the Examination of Water and Wastewater (APHA, 2005) [18]. pH values were measured in situ using a calibrated portable pH meter (HACH HQ40d, Hach Company, Loveland, CO, USA). The surface morphological evolution of CC and GAC substrates, particularly before and after microbial colonization, was characterized by field emission scanning electron microscopy (FE-SEM, Hitachi SU8010, Compass Technology Co., Ltd., Tokyo, Japan) operated at 5 kV accelerating voltage.

2.4. Method for Measuring Electrical Conductivity of Sludge

To validate that conductive material-mediated DIET pathways enhance electroactive bacteria enrichment in anaerobic sludge, this study employed a three-probe electrical conductivity measurement method to analyze the sludge's conductive properties. The anaerobic sludge was first centrifuged at 8000 rpm for 5 min, followed by three sequential wash cycles with 0.1 mol/L NaCl solution to remove surface-adhered impurities and mitigate external ionic interference. The washed anaerobic sludge was uniformly layered between two gold electrodes. Voltage gradients were applied to the electrodes using an electrochemical workstation (CH1030C, Chenhua Instruments, Shanghai, China). Steady-state ionic currents recorded under varying voltage levels were plotted to generate current–voltage curves for conductivity analysis.

The following equation represents the connection between the electrical conductivity (σ , S/m) and resistivity (ρ , $\Omega \cdot \text{m}$) of anaerobic sludge (Equation (1)):

$$\sigma = \frac{1}{\rho} \quad (1)$$

The resistivity (ρ) can be accurately derived from the slope of the current–voltage curve (Equation (2)), as follows:

$$\rho = \frac{R \times S}{L} \quad (2)$$

where R is the slope of the current–voltage curve (Ω); S is the cross-sectional area of the gold electrodes ($2.54 \times 10^{-6} \text{ m}^2$); L is the distance between the two gold electrodes ($0.5 \times 10^{-3} \text{ m}$).

2.5. Microbial Community Analysis

After the cow manure wastewater treatment system achieved steady-state operation, sludge adhering to the conductive material surfaces in each reactor was collected at the HRTs of 20 days, 8 days, and 4 days. Total genomic DNA was isolated from anaerobic sludge samples (collected from the UASB reactor) using the E.Z.N.A.[®] Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer's protocol. Extracted DNA quality was assessed via 1% agarose gel electrophoresis, with concentration and purity measured on a NanoDrop2000 spectrophotometer (Thermo Fisher, Waltham, MA, USA). Bacterial and archaeal 16S rRNA genes underwent PCR amplification with universal primers 338F-806R and modified archaeon-specific primers 524F10extF-Arch958RmodR, respectively. Replicate amplicons from identical samples were pooled, separated through 2% agarose gel electrophoresis, and recovered. Purification of recovered amplicons was performed using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA). Purified products were requantified employing both 2% agarose gel electrophoresis and a Quantus[™] Fluorometer (Promega, Madison, WI, USA). Sequencing libraries were prepared with the NEXTFLEX[™] Rapid DNA-Seq Kit and processed on an Illumina MiSeq PE300 platform (San Diego, CA, USA). Sequences were classified into operational taxonomic units (OTUs) using the Pyrosequencing Pipeline software (RDP release 11.5) (<https://warwick.ac.uk/fac/sci/lifesci/research/thermophyl/pipeline/> (accessed on 25 December 2023)).

2.6. Statistical Analysis

The statistical analysis was performed using Origin (2025), employing a *t*-test to compare a specific parameter across the experimental groups. Additionally, microbial community analysis was performed on the Majorbio cloud platform.

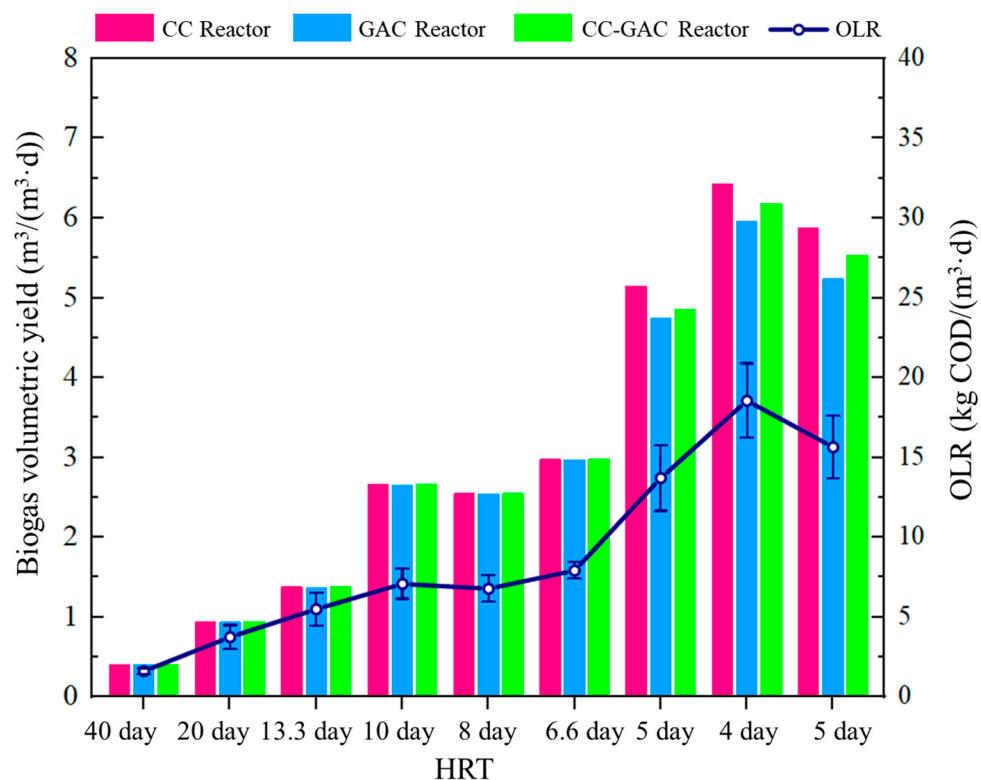
3. Results and Discussion

3.1. Performance of Different Reactors in AD Efficiency

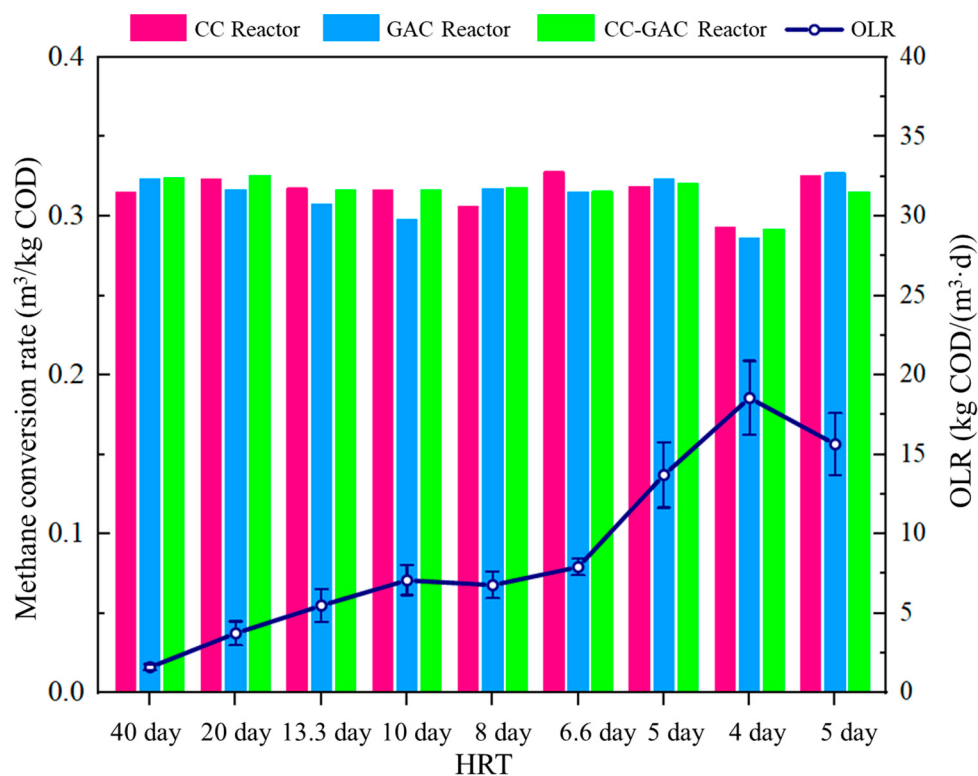
To visually represent the methane productivity of each reactor, operational data from each phase were converted into biogas volumetric yield and methane conversion rate, which served as indicators of resource utilization during cattle manure wastewater treatment. Throughout the first four operational phases, the biogas volumetric yield of all reactors increased steadily and eventually stabilized at $2.66 \text{ m}^3/(\text{m}^3 \cdot \text{d})$ (Figure 2a). When the HRT was reduced to 8 and 6.6 days, elevated ambient temperatures activated the sprinkler cooling systems in the barn, leading to lower influent COD concentrations. As a result, the OLR did not increase proportionally with the feed input, and no significant changes in biogas volumetric yield were observed, maintaining a level of $2.97 \text{ m}^3/(\text{m}^3 \cdot \text{d})$. Further reduction in the HRT to 5 days resulted in an increase in influent COD concentration, yielding an OLR of $13.73 \pm 2.06 \text{ kg COD}/(\text{m}^3 \cdot \text{d})$. Under these conditions, the biogas volumetric yield of the three reactors began to diverge, though no statistically significant differences were detected. The CC reactor achieved a yield of $5.15 \text{ m}^3/(\text{m}^3 \cdot \text{d})$, which was slightly higher than that of the GAC reactor ($4.75 \text{ m}^3/(\text{m}^3 \cdot \text{d})$) and the CC-GAC reactor ($4.85 \text{ m}^3/(\text{m}^3 \cdot \text{d})$). Throughout the operational period, all three reactors achieved complete removal of feed COD, with no observed accumulation of COD. The influent pH of the reactor was 8.12 ± 0.11 , while the effluent pH consistently remained within the range of 7.8–8.0. These conditions supported robust growth of methanogenic archaea.

To investigate the maximum treatment capacity of CCM-RF reactors, the HRT was further decreased to 4 days, driving the OLR above $20 \text{ kg COD}/(\text{m}^3 \cdot \text{d})$; however, the biogas volumetric yield of the reactors did not increase proportionally. Despite the HRT reduction to 4 days, which led to a decline in treatment efficiency for cattle manure wastewater, the pH remained stable at 8.0, with no acidification observed, indicating

stable reactor operation. When the HRT was readjusted to 5 days, the OLR recovered to 15.67 ± 1.95 kg COD/(m³·d), and the biogas volumetric yield exceeded that observed under the same HRT in the previous phase.



(a)



(b)

Figure 2. (a) Biogas volumetric yield, and (b) methane conversion rate of each reactor.

By comparing the average methane conversion rate across all reactors during each operational phase, all values exceeded $0.30 \text{ m}^3/\text{kgCOD}$ (Figure 2b). The highest methane conversion rate was observed in the CC reactor during the final operational phase, where under the HRT of 5 days and the OLR of $15.67 \text{ kg COD}/(\text{m}^3 \cdot \text{d})$, the methane conversion rate reached $0.32 \text{ m}^3/\text{kgCOD}$ and the steady-state production of methane reached at least $85 \text{ Nm}^3/\text{day}$. In this phase, 91.43% of the COD removed from the CC reactor was converted into methane.

In summary, all tested CCM-RF enhanced AD of cattle manure wastewater and improved methane productivity. Comparative analysis revealed that prior to the 6.6-day HRT phase, no significant differences in biogas volumetric yield were observed among the three reactors. As the HRT was reduced, discernible differences in biogas volumetric yield emerged, with performance ranking in the following descending order: the CC reactor exhibited the highest yield, followed by the CC-GAC reactor, and then the GAC reactor. Ordinary on site reactors typically achieve a biogas volumetric yield of $0.5\text{--}0.7 \text{ m}^3/(\text{m}^3 \cdot \text{d})$, with a corresponding methane conversion rate of $0.15\text{--}0.20 \text{ m}^3/\text{kgCOD}$. Compared to conventional technologies and ordinary on site reactors, the biogas yield per unit of manure wastewater increased by 1.5 to 2.0 times, the biogas volumetric yield rose by 8.0 to 10 times, and the methane conversion rate improved by 1.5 to 2.0 times [19].

3.2. Evolution of Internal Material Characteristics in the Reactor System

Upon completion of reactor operation, CC and GAC were retrieved to examine microbial colonization on their surfaces. SEM imaging revealed dense biofilms colonizing both CC and GAC, with biofilm thickness ranging from 15 to $45 \mu\text{m}$ (CC) and 8 to $25 \mu\text{m}$ (GAC) (Figure 3a). These results confirm that the high specific surface area and the electrical conductivity of the materials synergistically promoted biofilm development, further indicating their role as effective microbial habitats.

The sludge electrical conductivity in all reactors was monitored during operation (Figure 3b). The electrical conductivity of the raw sludge was measured at $2.56 \mu\text{S}/\text{cm}$. As the reactor operation progressed, the sludge electrical conductivity in all reactors gradually increased and stabilized, peaking at $17.02 \mu\text{S}/\text{cm}$. Moreover, the sludge electrical conductivity exhibited a significant positive correlation with the relative abundance of EET-capable genera, as quantified by 16S rRNA gene amplicon sequencing. These findings confirm that the electroactive bacteria, synergistically interacting with methanogens under the mediation of CCM-RF, established the DIET pathway for direct electron exchange, thereby significantly enhancing the treatment efficiency of cattle manure wastewater.

3.3. Microbial Community Structure Analysis

Archaeal genus dominance across reactors included *Methanothrix*, *c__Bathyarchaeia*, *Methanobacterium*, and *Methanosarcina* (Figure 4). The dominant methanogens in the system were acetoclastic methanogens, which accounted for 57–79% of the total methanogenic community. Their synergistic interactions with acetate-oxidizing electrotrophs facilitated IET, thereby further reinforcing their dominant status within the system [20]. Notable shifts in the methanogenic community were observed compared to the original inoculum: the relative abundances of *Methanothrix* and *Methanosarcina* increased significantly, whereas those of *Methanobacterium* and *Methanocorpusculum* decreased. These community changes were correlated with the addition of CCM-RF and the substrate composition of cattle manure wastewater, which highlights the critical interplay between conductive materials and microbial syntrophy.

c__Bathyarchaeia is abundantly detected in AD treating cow manure wastewater, where it plays foundational roles in methanogenesis via hydrogenotrophic, methylotrophic, and

acetoclastic pathways [21]. It amplifies methyl-coenzyme M reductase activity, boosting methane synthesis. This microbe degrades cellulose, lignin, and proteins during hydrolysis, specializing in hemicellulose/cellulose fermentation—likely fueled by manure’s high carbohydrate content [22,23]. Notably, *c__Bathyarchaeia* employs the acetyl-CoA pathway for autotrophic metabolism, potentially outcompeting hydrogenotrophic methanogens such as *Methanobacterium*, whose abundance dropped from 40% to below 10% under CCM-RF reactors. *Methanobacterium*, a hydrogenotrophic methanogen, reduces CO₂ via H₂/formate with DIET pathways and forms syntrophic relationships with organic acid oxidizers [24]. Its decline under CCM-RF suggests inhibition of hydrogenotrophic methanogenesis.

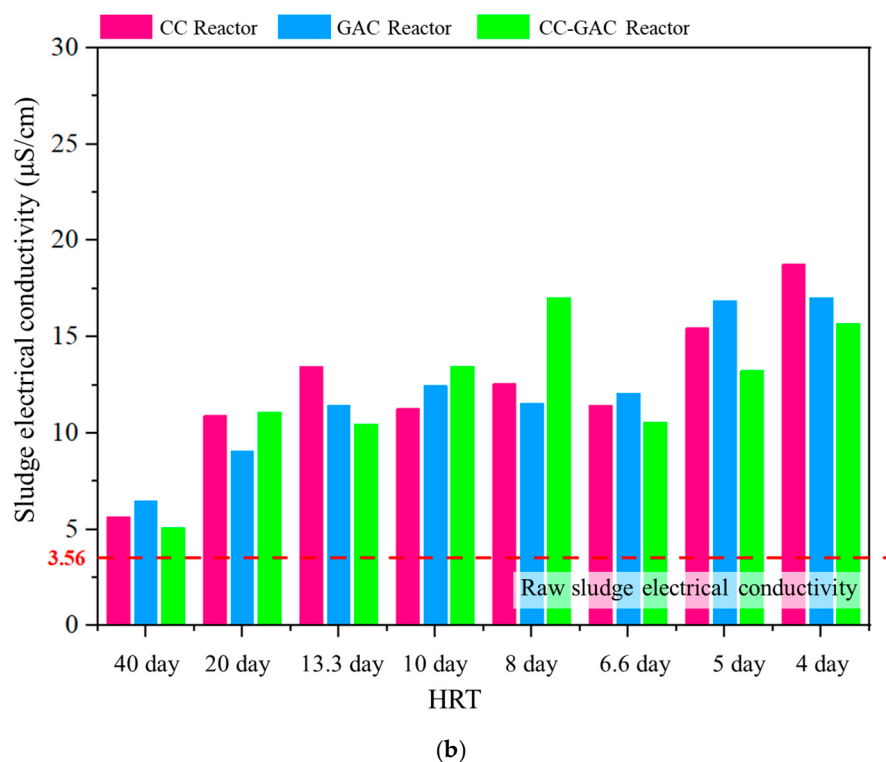
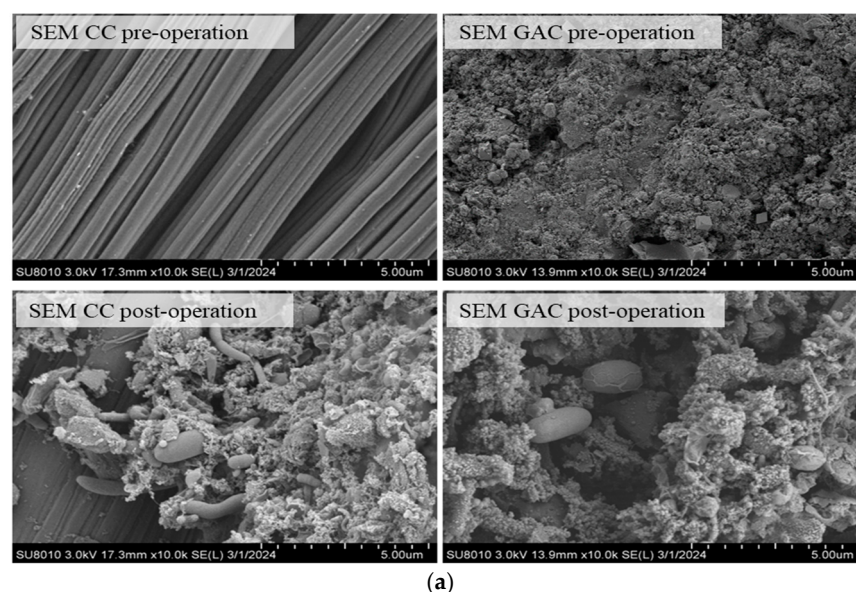


Figure 3. (a) SEM images of CC and GAC surface morphologies before/after reactor operation; (b) sludge electrical conductivity at each stage of the reactor.

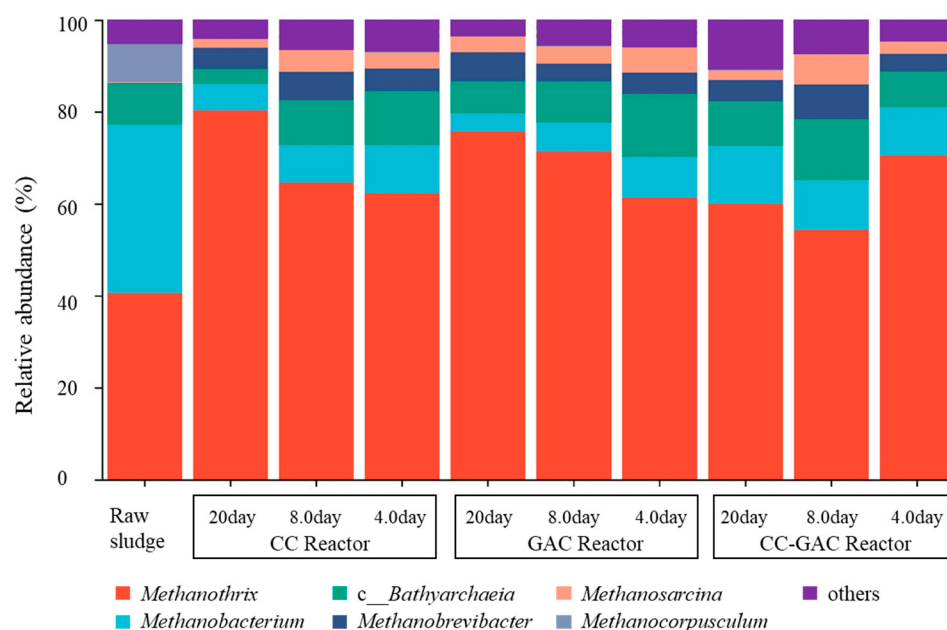


Figure 4. Relative abundance of dominant archaea genera at the genus level.

Previous studies demonstrate that *Methanotherix* enriches on CCM surfaces in AD, producing methane via DIET [25]. *Methanosarcina*, a mixotrophic methanogen, utilizes hydrogenotrophic, acetoclastic, and DIET pathways for methanogenesis [17]. Although a large inoculum of *Methanosarcina* was introduced during reactor startup, its relative abundance did not surpass that of *Methanotherix*, primarily due to the coexistence of indigenous *Methanotherix* in the cattle manure wastewater. A recent study reported similar findings, where the relative abundance of *Methanosarcina* significantly decreased after discontinuing propionate feeding in continuous-feed systems and was eventually replaced by other methanogenic communities [20]. Notably, despite its low relative abundance, *Methanosarcina* exhibited a marked increase compared to its baseline level in raw sludge, particularly in reactors amended with GAC, where its abundance gradually rose under high OLR and low HRT conditions. After adding CCM-RF to the AD system, different CCMs did not induce significant differences in archaeal community structure. However, the CCM-RF amendment predominantly enriched acetoclastic methanogens.

Based on bacterial genus abundance across reactors, dominant species included *Christensenellaceae_R-7_group* and *DMER64*, with minimal inter-reactor variability (Figure 5). *Christensenellaceae_R-7_group* exhibited strong cellulolytic activity, converting cellulose, hemicellulose, and lignin in cow manure into volatile fatty acids, dominating all tested systems [26]. Prevalent in cow manure wastewater, this genus secretes cellulases/hemicellulases to produce acetate/butyrate for methanogens, suggesting its origin from the manure feedstock [27]. *DMER64*, an electroactive microbe, facilitates interspecies hydrogen transfer and syntrophic butyrate oxidation, establishing DIET with methanogens in magnetite-mediated systems [28,29]. Its enrichment promotes symbiosis with methanogens, enhancing DIET and methane yields [30]. In CCM-RF reactors, *Anaerolineae* was enriched compared to original sludge, indicating its role in EET to methanogens via CCM. *Bacteroidetes_vadinHA17* degrades recalcitrant carbohydrates, aiding lignocellulose breakdown under low-OLR conditions [30]. As an electroactive species, it establishes DIET pathways with methanogens under CCM-RF [31]. The increased relative abundance of electroactive genera in CCM-RF reactors highlights enhanced DIET activity. These bacteria likely utilize CC/GAC as conductive matrices to transfer electrons from organic metabolism to methanogens, optimizing microbial synergy and methane production.

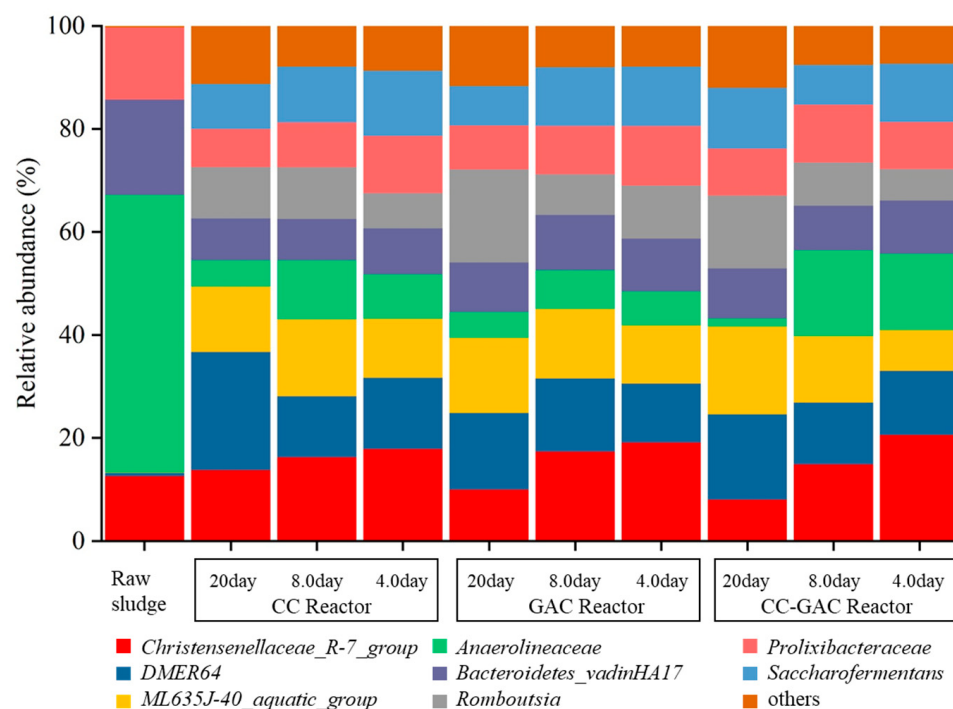


Figure 5. Relative abundance of dominant bacterial genera at the genus level.

3.4. DIET Metabolic Profiling

Current studies utilize conductive materials in AD to enhance DIET, optimizing microbial electron exchange between *Geobacter* and methanogens for improved methane production [32]. However, *Geobacter* was not enriched as a dominant genus in this study, consistent with prior research showing below 0.1% abundance in CC-supplemented landfill leachate digesters [33]. This may arise from two factors: high concentrations of recalcitrant organics in cow manure wastewater limiting *Geobacter* activity, and elevated salinity from bovine diets that are unfavorable for its survival.

Although *Geobacter* and *Sporanaerobacter* were not enriched in reactors treating cow manure wastewater, abundant electroactive bacteria (e.g., *DMER64*, *Anaerolineae*, and *Romboutsia*) were detected, collectively accounting for up to 25% abundance across reactors. Unlike *Geobacter*, which transfers electrons via conductive pili (e-pili) to methanogens like *Methanothrix*, these genera lack characterized e-pili structures [8]. However, conductive pilin-like proteins have been identified in diverse non-*Geobacter* bacteria [34]. Sludge electrical conductivity in CCM-RF reactors exceeded controls by up to 5 times, attributed to elevated e-pili expression from DIET-active microbial communities that enhance EET pathways.

Based on correlation and trend analyses of methanogenic archaeal genera under different OLR, *Methanothrix* and *Methanosarcina* dominated the methanogenic communities, while CCM-RF significantly altered the microbial community structure in the AD system (Figure 6). CCM-mediated DIET played a pivotal role in these processes. Electrons generated during metabolism by bacteria such as *DMER64* were directly transferred to *Methanosarcina* via CCM. These methanogens then utilized the electrons to reduce CO₂ into methane. Although a large inoculum of *Methanosarcina* was introduced during reactor startup, its relative abundance did not exceed that of *Methanothrix*. Notably, *Methanosarcina* maintained its activity under extreme conditions by utilizing diverse substrates for methanogenesis [35]. Furthermore, *Methanosarcina* alleviated inhibition caused by volatile fatty acid accumulation through the formation of multicellular aggregates, indicating that its proliferation represents a systemic adaptive response to propionate buildup.

under high OLR. This may explain why *Methanosarcina* generally exhibits low relative abundance in most AD systems. Under low acetate concentrations, *Methanothrix* outcompeted *Methanosarcina* due to its significantly higher affinity for acetate, thereby dominating the methanogenic archaeal community [36]. However, in reactors amended with GAC, the relative abundance of *Methanosarcina* progressively increased under high OLR and short HRT, offering critical insights for future strategies aimed at enriching *Methanosarcina*.

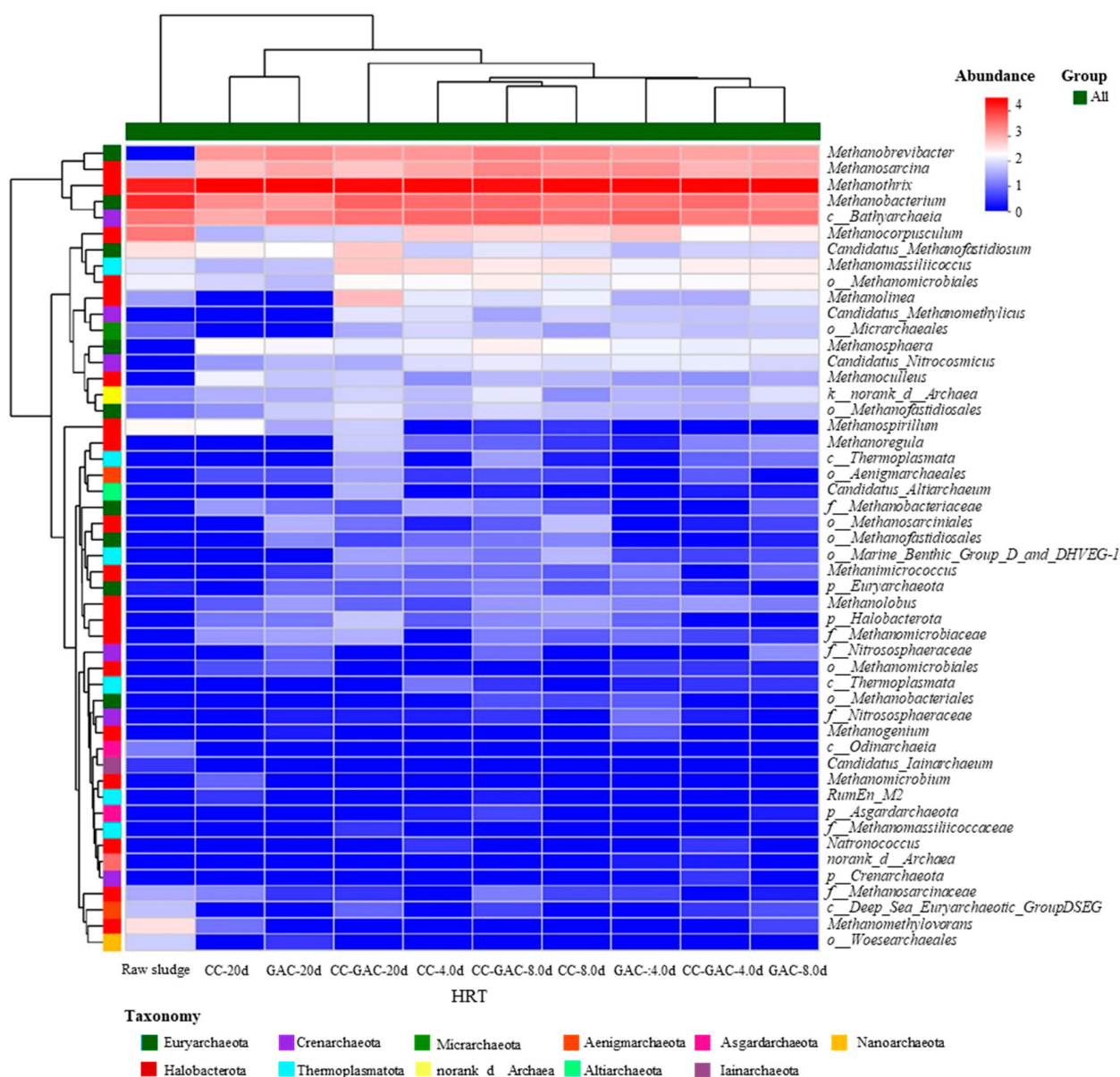


Figure 6. Correlation heatmap of methanogenic archaea genera under different OLR conditions.

3.5. Cost–Benefit Analysis

The cost–economic analysis of a pilot-scale cattle manure wastewater treatment system (working volume: 20 m³) demonstrated that the AD process enhanced with CCM-RF achieved a treatment capacity of 4 m³/d (1460 m³/year). The capital expenditure totaled EUR 21,930, covering reactor construction, conductive materials, and ancillary equipment. The annual operational expenditure was EUR 1377, which included riboflavin replenishment and energy consumption.

Compared to the conventional UASB reactor, the upgraded reactor exhibited an increase in biogas production of up to two-fold. Annually, the system produced 31,000 m³

of biogas, equivalent to 62,000 kW·h of electricity (based on 2.2 kW·h per m³ of biogas), generating EUR 3720 in revenue at an electricity price of EUR 0.06 per kW·h. With an income of EUR 2.5 per ton of treated wastewater and a net profit of EUR 1.6 per ton after accounting for operational costs, the technology achieved full capital recovery within 10 years. This approach effectively transforms waste treatment obligations into a sustainable revenue stream. The detailed calculation procedure is provided in the Supplementary Materials.

4. Conclusions

This study evaluated the enhancement effect on methane production via pilot-scale anaerobic reactors integrated with different CCM-RF configurations during the treatment of cattle manure wastewater. All systems exhibited improved AD performance and maintained stable operation at the OLR of 15.66 ± 1.95 kg COD/(m³·d), with a maximum OLR of 20 kg COD/(m³·d) and the HRT as short as 5 days. Among the configurations, the CC reactor outperformed the others, achieving a methane volumetric yield of 6.42 m³/(m³·d), which represents an eight-fold increase compared to conventional AD systems. The lifecycle cost–benefit analysis further demonstrated that the technology enables full recovery of capital expenditure within 10 years, thereby transforming wastewater treatment from a cost-incurring process into a profit-generating enterprise through integrated resource recovery.

Microbial community analysis revealed that CCM-RF addition primarily enriched acetoclastic methanogens, with *Methanothrix*, *Methanobacterium*, and *Methanosarcina* dominating across reactors. The sludge conductivity in the CCM-RF reactor was 6 times higher than that of raw sludge, further confirming that riboflavin-loaded carbon materials enhance DIET-driven methanogenesis. Although the high-inoculum *M. barkeri* had minimal impact on microbial community structure during startup, its relative abundance significantly increased compared to baseline levels in raw sludge. Notably, *M. barkeri* exhibited progressive proliferation under conditions of high OLR and short HRT, offering valuable insights for the development of future strategies aimed at enriching *Methanosarcina*.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/w17202967/s1>, Table S1. Construction expense calculation; Table S2. Operating expense calculation.

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Data Availability Statement: All data reported in this paper will be shared by the lead contact upon request. Requests for additional information should be addressed to the lead contact.

Conflicts of Interest: Author Qingtao Deng was employed by the company Zhongken Huashan Animal Husbandry Co., Ltd.; Author Jianyong Ma was employed by the company Inner Mongolia Gewei Huanneng Engineering Technology Co., Ltd.; Authors Haiyu Xu and Hongbin Wu were employed by the company Qinglin Chuangneng (Shanghai) Technology Co., Ltd. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

CCM-RF	Riboflavin-functionalized carbon-based conductive materials
DIET	Direct interspecies electron transfer
AD	Anaerobic digestion
<i>M. barkeri</i>	<i>Methanosarcina barkeri</i>
DSMZ	German Collection of Microorganisms and Cell Cultures
CC	Carbon cloth
GAC	Granular activated carbon
OLR	Organic loading rate
HRT	Hydraulic retention time
IET	Interspecies electron transfer
EET	Extracellular electron transfer
UASB	Upflow anaerobic sludge blanket
TS	Total solids
VS	Volatile solids
tCOD	Total chemical oxygen demand
OTUs	Operational taxonomic units

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